IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	:	
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Curran; *et al.*	:	Art Unit: 1656
	:	
Serial No. 10/078,927	:	Examiner: David Steadman
	:	
Filed: February 19, 2002	:	Atty Docket: SJ-01-0032
	:	
For: Cyclin Dependent Kinase 5 Phosphorylation of Disabled 1 Protein	:	

APPEAL BRIEF PURSUANT TO 37 C.F.R. §41.37

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

This revised Appeal from the rejection of claims 1, 4-8, 10-11, 13-15, 32 and 35 of the referenced application (published on November 21, 2002 as Pub. No. 2002/0172990) dated May 1, 2006 is in response to the Notification of Non-Compliant Appeal Brief issued on December 22, 2006. An Amendment under CRF §§ 1.111 and 1.121 to a non-final Office Action was originally filed on August 23, 2006 in which small portions of the text were missing, and then refiled on October 16, 2006 at the recommendation of the Electronic Business Office. New claims 36 – 40 were added in this Amendment. No action has been issued by the Examiner; however, it is expected the Amendment will be entered. The Notice of Appeal was also filed for this application on August 23, 2006, making this Appeal Brief due on October 23, 2006.

I. REAL PARTY IN INTEREST

The real party in interest in this application is St. Jude Children's Research Hospital by virtue of an assignment executed by both named inventors on February 18, 2002 and recorded in the U.S. Patent Office at Reel/Frame 012919/0525 (4 pages).

II. RELATED APPEALS AND INTERFERENCES

No other appeals or interferences are known to appellant, appellant's legal representative, or assignee St. Jude Children's Research Hospital which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF THE CLAIMS

There are a total of 40 claims in this application. Claims 2-3, 9, 12,16-31 and 33-34 have been canceled. Claims 1, 4-8, 10-11, 13-15, 32 and 35 are pending and stand rejected. New claims 36-40 were added in the Amendment filed August 23, 2006 after numerous attempts by the Appellants to modify the claims based on rejections of the Examiner failed to further prosecution. New claims 36 – 40 are broad claims similar to claims previously rejected by the Examiner. These prior rejections are addressed herein based on the assumption that they will be applied by the Examiner to these new claims. No claims have been allowed.

Claims 1, 4-8, 10-11, 13-15, 32 and 35 - 40 are on appeal. The claims on appeal are reproduced in their present form in the attached Claims Appendix.

IV. STATUS OF AMENDMENTS

An amendment to an outstanding non-final Office Action mailed May 1, 2006 was filed on August 23, 2006 and then refiled on October 16, 2006 when it was discovered that small portions of the text were missing. This Amendment has yet to be officially entered by the Examiner, but Appellants expect it to be entered and have incorporated it into the claims appearing in the attached appendix.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The claimed subject matter is based on the discovery that the Disabled 1 protein (Dab1) is a substrate for cyclin-dependent kinase (Cdk5) activity, and is selectively phosphorylated by

Cdk5 in vivo. Based on this discovery, an assay to determine Cdk5 activity by detection of Dab1 phosphorylation is provided in Claim 1. Page 4 lines 14 – 25; page 5 lines 1 – 4; page 20, lines 8 through page 21, lines 2; and page 21 lines 24 – 31 of the specification show that the assay is based on the identification of two serine amino acids in Dab1 that are selectively phosphorylated by Cdk5, serine 491 or 515, particularly serine 491. SEQ ID Nos: 4 and 5 were incorporated into the specification based on the fact that they are the sequences associated with the Genbank Accession numbers provided as part of the definition for human and mouse Dab1 proteins on page 4 lines 22 – 25 of the specification.

Further embodiments of the invention found in Claims 32, 35 and 38 relate to an assay based on immunoprecipitation of Dab1 using an antibody generated from an oligopeptide, SEQ ID No:3, that contains phosphorylated serine 491 (page 15, lines 16 – 21 and page 21; lines 4 – 31 of the specification).

A further embodiment found in Claim 37 relates to an assay to determine Cdk5 activity by detection of phosphorylated serine within a candidate sequence preferred by cdk5 activity (see page 5; lines 1-3 and page 20 lines 8-18 of the specification).

A further embodiment found in Claim 39 relates to the identification of a human or mouse Dab1 based on sequences found in GenBank Accession numbers (page 4 lines 22 – 25 of the specification).

A further embodiment found in Claim 40 relates to the identification of a Dab1 protein as a protein that contains SEQ ID NO:3. This is supported on page 15 lines 16 – 21 of the specification.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The following issues are to be reviewed:

1) Whether Claims 36 – 38 are properly rejected under 35 U.S.C. §112, first and second paragraphs in prior office actions as indefinite based on use of the terms "Cdk5" and "Dab1".
2) Whether Claims 36 - 38 were properly rejected under 35 U.S.C. §112, first paragraph in prior office actions for failure of the specification to describe a sufficient number of species to support a genus.
3) Whether Claims 36, 38 and 40 were properly rejected under 35 U.S.C. §112, second paragraph in prior office actions as indefinite based on use of the term "candidate sequence".
4) Whether Claim 39 was properly rejected under 35 U.S.C. §112, second paragraph in prior office actions as being indefinite for containing genbank accession numbers.
5) Whether Claim 40 was properly rejected under 35 U.S.C. §112, first paragraph in prior office actions for inserting new matter into the disclosure based on inclusion of SEQ ID NO:3 as a structural limitation.
6) Whether the amendment filed on April 25, 2005 is properly objected to under 35 U.S.C. §132(a), for introducing new matter into the disclosure of the invention and introducing the alleged new matter into the claims, thus necessitating their rejection on this ground.
7) Whether claims 1, 4-8, 10-11, 13-15, 32 and 35 are properly rejected under 35 U.S.C. §112, first paragraph for failure to comply with the written description requirement.

VII. ARGUMENTS

A. The terms Cdk5 and Dab1 were well known in the art at the time the present application was filed and adequately describe the invention

Claims 36 - 38

Examiner's Arguments

Throughout prosecution of the present application, the Examiner rejected claims reciting the terms "Cdk5" and "Dab1", now represented by Claims 36 – 38, under 35 USC § 112, first and second paragraphs asserting that these terms are unclear. The Examiner has stated that there is no "clear definition" of the terms "Cdk5" and "Dab1" in the specification and even though these terms may have been used in the art at the time of the invention, the definitions of the terms in the specification are not limited to those "Cdk5" and "Dab1" polypeptides that were known in the art at the time of the invention including genbank accession numbers 3288851 and 1771281. The Examiner stated that the specification fails to define which of the Dab1 and Cdk5 properties are necessary for inclusion of a cyclin-dependent kinase or a disabled-1 protein which is distinct in sequence from similar proteins that may share these characteristics.

Even though the Examiner acknowledged that Dab1 and Cdk5 were known in the art at the time of filing of the invention and further acknowledged that the claims were not drawn to the Dab1 or Cdk5 polypeptides themselves, he asserted that the terms must be limited to particular sequences to meet the requirement of definiteness.

Appellants' Arguments

There is a strong presumption that an adequate written description of the claimed invention is present in the specification. *In re Wertheim*, 541 F.2d 257, 191 U.S.P.Q. 90 (Ct. Cust. Pat. App. 1976); *see also* Manual of Patent Examining Procedure (MPEP) Sec. 2163, page 156, col. 1. To overcome this presumption, the Examiner bears the initial burden of presenting evidence or reasons why a person skilled in the art would not recognize that the written

description of the invention provides support for the claims. *In re Wertheim*, 541 F2d at 263-264; *see also* MPEP Sec. 2163, page 158, col. 2. Appellants do not believe the Examiner has met this burden in this case for the reasons set forth below.

The terms "Cdk5" and "Dab 1" were well known in the art as of the application filing date of February 19, 2002 and are described in the specification in a manner consistent with these meanings. The specification defines "Cdk5" as "a protein with serine/threonine kinase activity that is structurally homologous to the mitotic cyclin dependent kinases"(p. 4) and defines "Dab1" as "an intracellular adapter protein that is phosphorylated by Cdk5 activity and by reelin tyrosine kinase activity" (p. 4). Appellants also include genbank accession numbers for human and mouse in the definition of Dab1 and human, mouse and rat genbank accession numbers for Cdk5. Appellants also included numerous references in the specification and during prosecution showing prior scientific publications that describe properties of Dab1 and Cdk5 which distinguish them from other cyclin dependent kinases and closely related Dab proteins.

These terms are in fact creations of the art used to denote, in each case, a class of proteins with a unique set of features that allowed them to be grouped together and distinguished from other proteins, even those that are closely related. Furthermore, Dr. Thomas Curran, a co-inventor of the present application and a person of skill in the art provided an expert declaration stating that "Cdk5", "Dab1" and "Cdk5 serine kinase activity" were well known terms in the art at the time the application was filed (see Evidence Appendix, Exhibit 1). Because these terms were well known in the prior art, an exhaustive description does not need to be reproduced in the specification and in fact is preferably omitted according to *Hybritech, Inc. v. Monoclonal Antibodies, Inc.* 802 F.2d 1367, 1384, 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986.)

Appellants do not rely upon the primary structure; i.e. the amino acid sequence, of any of these proteins to impart patentability upon the claimed compositions. Instead Appellants properly rely on the knowledge of these structures to supplement the description of the novel and unobvious aspects of the invention in the specification. Recitation of the primary structure of

each member of this group of proteins would be redundant to knowledge available in the prior art and is not necessary.

The invention is based on the discovery that Dab1 is specifically phosphorylated by Cdk5. Cdk5 activity is tightly controlled by its regulator, p35, making Cdk5 activity difficult to determine based on levels of Cdk5 present. Furthermore, a substrate which is selectively phosphorylated by Cdk5 had not heretofore been identified. The discovery that Dab1 is specifically phosphorylated on serine within a preferred candidate sequence by Cdk5 is the basis for the invention. The invention is not based on the novelty or nonobviousness of Cdk5 or Dab1, but rather on the special relationship between the two as taught for the first time in the present application. The invention claimed in the present application is directed to a unique method for determining Cdk5 serine kinase activity based on this special relationship.

In *Falkner v. Inglis*, ---F.3d---, 2006 WL 1453040, Slip No. 05-1324 (Fed. Cir. May 26, 2006) the Court of Appeals for the Federal Circuit (CAFC) agreed with the Board of Patent Appeals and Interferences (BPAI) that the poxvirus-based vaccines described in the Inglis applications were adequately described and enabled even though the specification contained no poxvirus sequences or specific examples for making a poxvirus vaccine or the phrase "incorporated by reference". Likewise, in *Capon et al. v. Eshar et al.,* Nos. 03-14480, 1481 (Fed. Cir. August 12, 2005), the CAFC reversed the BPAI and found that claims to chimeric genes composed of pieces of known genes did not need to recite the known gene sequences to satisfy the written description requirement.

As Appellants have argued in the present case regarding the incorporation of "Dab1" and "Cdk5" sequences, the CAFC found that at the time of filing of the earliest Inglis application, the poxvirus genome was well known to those of ordinary skill in the art as evidenced by publication of the genome in professional journals. In view of the well known nature of the poxvirus genome, neither the BPAI or the CAFC found it necessary for the applicant to incorporate the well known poxvirus genome into the specification by reference or otherwise. Indeed, the CAFC

noted that omission of such redundant information from the specification is preferred, reiterating the familiar adage that "[a] patent need not teach, and preferably omits, what is well known in the art." citing *Spectra-Physics, Inc. v. Coherent, Inc.*, 827 F.2d 1524, 1534 (Fed. Cir. 1987). The terms "Dab 1" and "Cdk5" were well known in the art at the time the present application was filed and, according to the reasoning used by the CAFC in *Falkner v Inglis* and *Capon v Eshar*, adequately describe the invention in compliance with 35 U.S.C. §112, first and second paragraphs. Therefore, Appellants respectfully submit that previous rejections made under 35 USC § 112, first and second paragraphs are improper and should not be applied to new claims 36 - 38.

B. The disclosed species are representative of the entire genus

Claims 36 - 38

Examiner's Arguments

In prior Office Actions, the Examiner rejected claims under 35 U.S.C. § 112, first paragraph for failure to describe a sufficient number of species to recite a genus for Cdk5 or Dab1. The Examiner asserted that the genera encompass widely variant species with respect to structure and that the three genbank nos. provided for Cdk5 and two genbank nos. provided for Dab1 were insufficient representatives of the genus. The Examiner asserted that other than the two representative species of Dab1 polypeptides, the specification failed to disclose any other additional representative species of the genus. The Examiner maintained that the disclosure of the two representative species of Dab1 polypeptides is insufficient to be representative of the attributes and features of all species encompassed by the recited genus of Dab1 polypeptides.

The Examiner maintained that the alleged novel relationship claimed by Appellants, i.e., the phosphorylation of Dab1 by Cdk5, has not been shown in all organisms that express "Cdk5" and "Dab1" polypeptides. The Examiner asserted that there is no disclosure in the specification or in the prior art of a structure-function correlation between the members of the respective genus

of Cdk5 or Dab1 polypeptides such that by the mere recitation of "Cdk5" or "Dab1" one of skill can visualize the structures of all members of the respective genus.

Appellants' Arguments

"Any assertion by the Patent Office that the enabling disclosure is not commensurate in scope with the protection sought must be supported by evidence or reasons substantiating the doubts so expressed." *In re Dinh-Nguten and Stenhagen*, 181 USPQ 46, 47 (Ct. Cust. & Pat. App. 1974); *see also In re Wright*, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993); *In re Armbruster*, 185 USPQ 152, 153 (Ct. Cust. & Pat. App. 1975); *In re Bowen*, 181 USPQ 48 (Ct. Cust. & Pat. App. 1974); *In re Hawkins*, 179 USPQ 157, 162 (Ct. Cust. & Pat. App. 1973). Even at the request of Appellants throughout prosecution, the Examiner provided no scientific rational or evidentiary support for the assertion that the disclosed Cdk5/Dab1 relationship is peculiar to a single species. In the absence of such support, Appellants submit that the Examiner has failed to carry his burden for establishing prima facie nonenablement.

Even though this rejection does not need to be affirmatively rebutted because it is not supported by any evidence or reasoning, Appellants have explained the enabling nature of the disclosure for all species that harbor Cdk5 and Dab1 proteins. Example 1 of the specification provides results obtained in a mouse model, which show Cdk5 specifically phosphorylates Dab1 at serine 491. Appellants provided evidence showing Dab1 phosphorylation on serine 491 is Cdk5 activity-dependent in a rat model as well (see Evidence Appendix, Exhibit 2). Appellants also provided sequence alignments for mouse, rat and human Cdk5 and Dab1 (which were all in the public domain at the time the present application was filed) showing that both proteins are highly conserved among the three species (see Evidence Appendix, Exhibit 3). Appellants also showed the Dab1 sequence alignment between mouse and human is 96% identical, mouse and dog is 90% identical, mouse and bird is 89% identical, mouse and cow is 84% identical and mouse and zebrafish is 66% identical (see Evidence Appendix, Exhibits 4 - 8).

Appellants have provided examples for two representative species and have provided support showing that Dab1 and Cdk5 are highly conserved proteins in many different species. In Falkner v. Inglis, ---F.3d---, 2006 WL 1453040, Slip No. 05-1324 (Fed. Cir. May 26, 2006) the CAFS states:

> A claim will not be invalidated on section 112 grounds simply because the embodiments of the specification do not contain examples explicitly covering the full scope of the claim language. That is because the patent specification is written for a person of skill in the art, and such a person comes to the patent with the knowledge of what has come before. Placed in that context, it is unnecessary to spell out every detail of the invention in the specification; only enough must be included to convince a person of skill in the art that the inventor possessed the invention and to enable such a person to make and use the invention without undue experimentation.

The specification in the Inglis application focused on herpesvirus; it did not show specific examples of the claimed invention as it related to a poxvirus. However, the CAFS held that a person skilled in the art was capable of taking the claimed invention and applying it to a poxvirus. Likewise, a person of skill in the art, would have been able to identify a Dab1 and Cdk5 protein from any desired species and determine if Dab1 was phosphorylated on a serine within a preferred sequence as taught in the present application.

Having shown examples of how specific Dab1 phosphorylation can be used as a proxy for Cdk5 activity in representative species, and showing these proteins are highly conserved among species, Appellants submit that one of skill in the art would have no problem using this method to detect the activity of any Cdk5 within other species. Accordingly, Appellants respectfully submit that previous rejections made under 35 U.S.C. § 112, first paragraph for failure to describe a sufficient number of species were improper and should not be applied to new claims 36 - 38.

C. The term "candidate sequence preferred by cdk5" is definite Claims 36, 38 and 40

Examiner's Arguments

The Examiner rejected prior claims for use of the term "candidate sequence preferred by cdk5 activity" under 35 U.S.C. 112, second paragraph as being indefinite. The Examiner acknowledged the definition of the term "candidate sequence" at page 5 of the specification. However, the Examiner asserted this definition provides no indication as to the scope of those candidate sequences that are "preferred" by a "cdk5 activity" and that the term remained indefinite. The Examiner maintained that this term is indefinite in view of the indefiniteness of the term "cdk5 activity" and that the scope of candidate sequences are those that are "preferred" by cdk5 activity. It is unclear from the specification and the claims as to whether all sequences that have a serine followed by proline at the +1 position and a lysine in the +3 position are those that are "preferred" by a "Cdk5 activity" or whether only a subset of those sequences that have a serine followed by proline at the +1 position and a lysine in the +3 position are meant to be encompassed as being sequences that are "preferred" by "cdk5 activity."

Appellants' Arguments

Appellants maintain that the specification specifically defines a "candidate sequence" as a sequence of amino acids which contains a serine followed by a proline in the +1 position and a lysine in the +3 position, the serine being a preferred site for Cdk5 activity (Songyang et al., Mol Cell Biol, 16:6486-6493, 1996, Evidence Appendix, Exhibit 9). Songyang et al. teach that this sequence is a distinct optimal peptide substrate for the Cdk5 kinase. Furthermore, on page 20 lines 8-18 of the specification, Appellants predicted murine Dab1 serines 491 and 515 to be Cdk5 phosphorylation sites based on sequence analysis and then conducted experiments to show their prediction was true.

Appellants have shown the terms "Cdk5" and "Dab1" were well defined and were well known by a person of skill in the art at the time of filing. Dr. Thomas Curran, a co-inventor of the present application and a person of skill in the art stated in his expert declaration filed in the April 25, 2005 response that "Cdk5 serine kinase activity" was well understood and used

extensively in the art at the time the application was filed (see Evidence Appendix, Exhibit 1). It was well known in the art that serine kinase activity refers to the ability of a protein (in this case Cdk5) to phosphorylate a serine. A person of skill in the art knows the meaning of "cdk5 serine kinase activity" and the specification defines what constitutes a candidate sequence in a manner that allows a skilled artisan to routinely identify such sequences. Appellants discuss the definition of a candidate sequence and show how they determined that the serines within the two candidate sequences found in the carboxy terminal domain of Dab1 were phosphorylated by cdk5 serine kinase activity.

Appellants respectfully submit that rejections made under 35 USC § 112, second paragraph were improper and should not be applied to the use of the term "candidate sequence" in Claims 36, 38 and 40.

D. Genbank accession numbers are definite

Claim 39

Examiner's Arguments

The Examiner rejected previous claims containing genbank accession numbers as being indefinite. The Examiner asserted that it is well known to one of skill in the art that sequence accession numbers are updated by modifying the sequence of a particular accession number. The Examiner stated that there is no way to know with certainty that the sequence of genbank accession numbers will not change. Genbank accession numbers are not static, but can change by revision. The Examiner provided an example of the revision history for Accession Number X761041, a protein unrelated to this application (see Evidence Appendix, Exhibit 10).

Appellants' Arguments

The U.S. Patent Office has previously accepted the use of genbank accession numbers in claims to refer to biological sequence information known and available in the prior art. For example, U.S. Patent No. 6,770,742, issued August 3, 2004 claims a fibroblast growth factor

receptor-4 by reference to a genbank accession number. The FGFR-4 sequence is not listed in the sequence listing, thus there is no SEQ ID qualifier for the FGFR-4 receptor. Furthermore, the revision history for this genbank accession number shows that it was revised no less than 7 times. Additional granted patents in which the patentee was allowed to refer to nucleotide or amino acid sequences according to genbank numbers in the claims include, but are not necessarily limited to, U.S. Patent Nos. 6,949,342; 6,943,006; 6,890,572 and 6,667,065.

Since there appears to be no per se rule against the use of genbank accession numbers in claims, Appellants ask that the use of genbank numbers be allowed in this instance where the sequence itself does not go to the heart of the invention, but is simply useful information for understanding the invention.

In a copy of the revision history for Accession Number X761041 provided by the Examiner in the Office Action dated August 22, 2005 (see Evidence Appendix, Exhibit 10), each date the accession number was revised is clearly shown. Furthermore, a link is provided so that one can review the contents of the accession number for each date prior to a revision. Since a person of skill in the art knows the filing date of the present application, that person can easily access the sequence in genbank that was known at that time. Therefore, the use of genbank accession numbers in the claims is clear and definite. Furthermore, any changes that might be made to the Cdk5 or Dab1 sequences would not be expected to change the characteristics of these proteins that are important in the context of the claimed invention.

Appellants respectfully submit that rejections made under 35 USC § 112, second paragraph, based on the assertion that the use of genbank numbers renders the claims indefinite, are improper and should not be applied to Claim 39.

E. The use of SEQ ID NO:3 as a structural limitation is not new matter

Claim 40

Examiner's Arguments

In an attempt to address the Examiner's rejection of previous claims stating that a genus requires a precise definition, such as structure, formula or chemical name of the claimed subject matter to sufficiently distinguish it from other materials, Appellants amended the claims to require that the Dab1 protein include SEQ ID NO:3. Support for SEQ ID NO:3 can be found on page 3, lines 25 – 29 and page 15, lines 16-17.

The Examiner rejected the claims that incorporated SEQ ID NO:3 as a structural limitation for Dab1 proteins under 35 U.S.C. 112, first paragraph for inserting new matter. The Examiner asserted that while the disclosure provides support for the peptide of SEQ ID NO:3, it fails to support the recited genus of Dab1 polypeptides comprising SEQ ID NO:3. The Examiner maintained that while all members of the genus of Dab1 polypeptides comprise the structural feature of the 14 amino acid peptide of SEQ ID NO:3, this structural feature does not constitute a "substantial portion" of the genus of recited Dab1 polypeptides. Thus, the Examiner maintained the specification failed to adequately describe the claimed invention.

Appellants' Arguments

SEQ ID NO:3 comprises 14 amino acids found in the c-terminal portion of the Dab1 protein in several different species including mice, rats, humans, birds, dogs and cows. Proteins other than Dab1, even closely related proteins such as Dab2, do not share this sequence. SEQ ID NO:3 is provided as a common structural reference for the genus of Dab1 proteins to supplement the distinguishing features of Dab1 noted in the specification. A peptide having the sequence of SEQ ID NO:3, as shown in the specification, was used as an antigen to generate an antibody that binds to Dab1. The use of this peptide as an antigen reveals to one of skill in the art that this is a sequence that is characteristic of Dab 1 and useful for distinguishing Dab1 from other proteins. Therefore, inclusion of SEQ ID NO:3 in the claims as a feature of Dab 1 is fully supported by the specification and is not new matter.

Appellants respectfully submit that the rejection made under 35 U.S.C. § 112, first paragraph, for inserting new matter, should not be applied to Claim 40.

F. Introducing sequence information based on genbank accession numbers found in the specification without the words "incorporated by reference" specifically stated in the specification is not introducing new matter

Claims 1, 4 – 8, 10 – 11, 13 – 15, 32 and 35

The Examiner objected to the amendment filed April 25, 2005 under 35 U.S.C. 132(a) for introducing new matter into the disclosure. The Examiner asserts that according to MPEP § 608.01(p) incorporation by reference of material in a non-patent document "must be set forth in the specification and must: (1) Express a clear intent to incorporate by reference by using the root words "incorporat(e)" and "reference" (e.g., "incorporate by reference"); and (2) Clearly identify the referenced patent, application, or publication." See 37 § 1.57(b). Furthermore the Examiner states that MPEP § 608.01(p) further states, "[i]f a reference to a document does not clearly indicate an intended incorporation by reference, examination will proceed as if no incorporation by reference statement has been made and the Office will not expend resources trying to determine if an incorporation by reference was intended."

The Examiner rejected Claims 1, 4-8, 10-11, 13-15, 32 and 35 as failing to comply with the written description requirement. The Examiner asserts that the claim(s) contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

Prior to making the present objection and rejection, the Examiner previously determined that SEQ ID NOs: 4 and 5 were intended to be incorporated by reference based on the inclusion of the appropriate genbank numbers. The Examiner withdrew an objection in the August 22, 2005 Office Action, stating that the disclosed GenBank Accession Number in the specification is considered to be an inherent "incorporation by reference", and proceeded to examine the claims based on the inclusion of SEQ ID NO:4 (mouse Dab1). On page 9 of the Office Action dated

February 21, 2006, the Examiner allowed a claim that incorporated SEQ ID NO:4 and indicated that 2 other claims, one that incorporated SEQ ID NO:4 and another that incorporated SEQ ID NO:5, would be allowed if written in independent form. However, in the Office Action dated May 1, 2006, the Examiner reconsidered this determination and rejected the claims citing 37 C.F.R.§1.57 and MPEP § 608.01(p). The Examiner asserted the originally filed disclosure does not provide support for SEQ ID NO:4 and SEQ ID NO:5 which are included in Claims 1, 32 and 35 based on the fact that the root words "incorporate" and "reference" do not appear in the specification. According to the Examiner, even though the genbank accession numbers are included in the definition of Dab1, this is insufficient support for incorporating the sequences associated with these accession numbers (i.e. SEQ ID Nos. 4 and 5) because the specification does not specifically state the accession numbers are to be incorporated by reference.

Appellants respectfully disagree with the preceding objection and rejection of the Claims. The regulation cited to support this rejection, 37 C.F.R.§1.57, was added on Sept. 21, 2004 and became effective Oct. 21, 2004, well after the February 19, 2002 filing date of the present application. Furthermore, MPEP § 608.01(p) was not amended until Oct. 21, 2004 to include the language stated in the above objection by the Examiner. A patent application should not be faulted for failing to adhere to rules established well after its filing date.

However, even if Appellants are held to this standard, 37 C.F.R. § 1.57 (g)(1) does allow correction to comply with paragraph (b)(1) of this section if the application as filed clearly conveys an intent to incorporate the material by reference. 37 C.F.R. § 1.57 (g)(2) states that [a] correction to comply with paragraph (b)(2) of this section is permitted for material that was sufficiently described to uniquely identify the document.

On page 4, lines 24-25 of the specification, Appellants specifically define Dab1 proteins as including proteins cloned from genbank accession numbers 3288851 and 1771281. As Appellants argued earlier in its response dated June 16, 2005 to the Examiner's assertion made in the Advisory Action mailed May 18, 2005, including these genbank numbers as part of the

definition of Dab1 reflects Appellants' intent for these publications to be incorporated by reference. Appellants also indicated during prosecution that the sequences incorporated into the specification were the sequences found in the genbank accession numbers at the time of filing of the application. Thus, Appellants have fulfilled the requirements of 37 § 1.57 (g)(1) and (g)(2).

In light of the arguments presented above, Appellants have overcome the objection and rejection of Claims 1, 4-8, 10-11, 13-15, 32 and 35.

For the foregoing reasons, Appellants believe that the Examiner's rejections of Claims 1, 4-8, 10-11, 13-15, 32 and 35 – 40 are erroneous and reversal of all outstanding rejections is therefore respectfully requested.

Respectfully submitted,

Shawn A. Hawkins

Shawn Annette Hawkins
Agent for Appellants
Registration No. 50,318

St. Jude Children's Research Hospital
332 North Lauderdale
Memphis, TN 38105-2794
Telephone: 901-495-2751

Date: January 18, 2007

VIII. CLAIMS APPENDIX

Claim 1 A method for detecting cyclin dependent kinase 5 (Cdk5) serine kinase activity in a biological sample, which method comprises determining whether Disabled 1 protein (Dab1) in said sample is phosphorylated on a serine selected from the group consisting of a serine at position 491 of the polypeptide encoded by SEQ ID NO:4 or SEQ ID NO:5 and a serine at position 515 of the polypeptide encoded by SEQ ID NO:4 or SEQ ID NO:5, wherein phosphorylation of Dab1 on said serine indicates the presence of active Cdk5 in said sample.

Claim 4 The method of claim 1 wherein said biological sample is isolated from an organism selected from the group consisting of mouse and human.

Claim 5 The method of claim 1 wherein said biological sample is isolated from the group consisting of brain and blood.

Claim 6 The method of claim 1 wherein said biological sample is isolated from a cell culture.

Claim 7 The method of claim 1 wherein said Dab1 phosphorylation occurs *in vivo*.

Claim 8 The method of claim 1 which comprises immunoprecipitating said Dab1 from said biological sample prior to said determining step using an antibody that binds to Dab1 phosphorylated and unphosphorylated on said serine.

Claim 10 The method of claim 1 wherein Dab1 phosphorylation is determined using an antibody that binds to Dab1 only when it is phosphorylated on said serine.

Claim 11 The method of claim 10 wherein said antibody is raised against the polypeptide fragment TPAPRQSS(PO_4)PSKSSA (SEQ ID NO:3 which contains a phosphate group on the 8th amino acid).

Claim 13 The method of claim 10 wherein said antibody is polyclonal.

Claim 14 The method of claim 10 wherein said antibody is monoclonal.

Claim 15 The method of claim 10 wherein Dab1 phosphorylation is determined by using techniques consisting of radioimmunoassay, ELISA, "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitation reactions, immunodiffusion assays, in situ immunoassays, western blots, precipitation reactions, agglutination assays, complement fixation assays, immunofluorescence assays, protein A assays, immunoelectrophoresis assays, mass spectrometry and antibody array.

Claim 32 A method for detecting cyclin dependent kinase 5 (Cdk5) serine kinase activity in a biological sample, which method comprises immunoprecipitation of mouse Dab1 encoded by the sequence set forth in SEQ ID NO:4 from said biological sample; contacting the immunoprecipitated Dab1 with a phosphoantibody generated using SEQ ID NO:3 having a phosphorylated serine at position 8 of SEQ ID NO:3 as an antigen; detecting binding of the phosphoantibody to serine 491 of said Dab1, wherein binding of the phosphoantibody to serine 491 of said Dab1 in such biological sample indicates the presence of Cdk5 serine kinase activity in said sample.

Claim 35 A method for detecting cyclin dependent kinase 5 (Cdk5) serine kinase activity in a biological sample, which method comprises immunoprecipitation of human Dab1 encoded by the sequence set forth in SEQ ID NO:5 from said biological sample; contacting the immunoprecipitated Dab1 with a phosphoantibody generated using SEQ ID NO:3 having a phosphorylated serine at position 8 of SEQ ID NO:3 as an antigen; detecting binding of the phosphoantibody to serine 491 of said Dab1, wherein binding of the phosphoantibody to serine 491 of said Dab1 in such biological sample indicates the presence of Cdk5 serine kinase activity in said sample.

Claim 36 A method for detecting cyclin dependent kinase 5 (Cdk5) serine kinase activity in a biological sample, which method comprises determining whether the carboxy

terminal domain of Disabled 1 protein (Dab1) in said sample is phosphorylated on a serine within a candidate sequence preferred by cdk5 activity, wherein phosphorylation of Dab1 on said serine indicates the presence of active Cdk5 in said sample.

Claim 37 The method of claim 36 wherein said serine is selected from the group consisting of a serine corresponding to position 3 of QSSPSK (SEQ ID NO:1), such position being determined by alignment of Dab1 with reference to amino acid positions of SEQ ID NO:1 and a serine at position 21 of SSASHVSDPTADDIFEEGFESPSK (SEQ ID NO:2), such position being determined by alignment of Dab1 with reference to amino acid positions of SEQ ID NO:2.

Claim 38 A method for detecting cyclin dependent kinase 5 (Cdk5) serine kinase activity in a biological sample, which method comprises immunoprecipitation of Dab1 from said biological sample; contacting the immunoprecipitated Dab1 with a phosphoantibody generated using SEQ ID NO:3 having a phosphorylated serine at position 8 of SEQ ID NO:3 as an antigen; detecting binding of the phosphoantibody to a serine within a candidate sequence preferred by cdk5 activity in the carboxy terminal domain of said Dab1, wherein binding of the phosphoantibody to said serine of said Dab1 in such biological sample indicates the presence of Cdk5 serine kinase activity in said sample.

Claim 39 A method for detecting cyclin dependent kinase 5 (Cdk5) serine kinase activity in a biological sample, which method comprises determining whether Disabled 1 protein (Dab1) in said sample is phosphorylated on a serine selected from the group consisting of a serine corresponding to position 491 of the amino acid sequence encoded by the nucleotide sequence of GenBank Accession number 1771281 and a serine corresponding to position 515 of the amino acid sequence encoded by the nucleotide sequence of GenBank Accession number 1771281,

wherein phosphorylation of Dab1 on said serine indicates the presence of active Cdk5 in said sample.

Claim 40. A method for detecting cyclin dependent kinase 5 (Cdk5) serine kinase activity in a biological sample, which method comprises determining whether the carboxy terminal domain of Disabled 1 (Dab1) protein comprising SEQ ID NO:3 in said sample is phosphorylated on a serine within a candidate sequence preferred by cdk5 kinase activity, wherein phosphorylation of Dab1 on said serine indicates the presence of active Cdk5 in said sample.

IX. *EVIDENCE APPENDIX*

Content

Exhibit 1 Curran declaration included in Appellants' amendment and response filed April 25, 2005 (see pages 6 and 12 of this appeal).

Exhibit 2 Rat results included in Appellants' amendment and response filed April 25, 2005 (see page 9 of this appeal).

Exhibit 3 Sequence alignment for rat, mouse and human included in Appellants' amendment and response filed April 25, 2005 (see page 9 of this appeal).

Exhibit 4 Sequence alignment comparing mouse and human Dab1 proteins, included in Appellants' amendment and response filed April 3, 2006 (see pages 9 and 10 of this appeal).

Exhibit 5 Sequence alignment comparing mouse and dog (Canis familiaris) Dab1 proteins, included in Appellants' amendment and response filed April 3, 2006 (see pages 9 and 10 of this appeal).

Exhibit 6 Sequence alignment comparing mouse and bird (Gallus galus) Dab1 proteins, included in Appellants' amendment and response filed April 3, 2006 (see pages 9 and 10 of this appeal).

Exhibit 7 Sequence alignment comparing mouse and cow (Bos taurus) Dab1 proteins, included in Appellants' amendment and response filed April 3, 2006 (see pages 9 and 10 of this appeal).

Exhibit 8 Sequence alignment comparing mouse and zebrafish (Banio rerio) Dab1 proteins, included in Appellants' amendment and response filed April 3, 2006 (see pages 9 and 10 of this appeal).

Exhibit 9 Songyang et al., Mol Cell Biol, 16:6486-6493, 1996, included in the Information Disclosure filed March 19, 2002 (see page 11 of this appeal).

Exhibit 10 Examiner's accession number revision document, submitted in the office action mailed August 22, 2005 (see page 13 of this appeal).

Evidence Appendix
Exhibit 1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	:	
	:	
Curran; *et al.*	:	Art Unit: 1652
	:	
Serial No. 10/078,927	:	Examiner: David J. Steadman
	:	
Filed: February 19, 2002	:	Atty Docket: SJ-01-0032
	:	
For: Cyclin Dependent Kinase 5 Phosphorylation of Disabled 1 Protein	:	

Mail Stop Amendment
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132
By
Dr. Thomas Curran

Sir:

I, Thomas Curran, do hereby declare and say as follows:

1. I am skilled in the art of the field of the invention of the above-referenced application. I have a PhD from Imperial Cancer Research Fund Laboratories and University College London, UK. I have been engaged in the study of development neurobiology for 21 years. I have been a faculty member and the Chair of the Department of Developmental Neurobiology at St. Jude Children's Research Hospital since 1995. The department consists of 8 faculty members and more than 50 employees. I am a past president of the American Association for Cancer Research and currently serve on the National Cancer Institute Board of Scientific Advisors. I am the current editor of Developmental Brain Research.

2. I am a co-inventor of the above-referenced application.

3. At the time of filing of the present invention on February 19, 2002, a person of skill in the art was capable of easily determining the scope of proteins that were encompassed by the term "Cdk5". Cdk5 was distinguishable from other proteins, including other closely related proteins, such as Cdc2, Cdk2, Cdk4 and Cdk6. Unlike other cdks, Cdk5 kinase activity has been observed only in the adult brain. The expression and activity of Cdk5 increases as increasing numbers of cells exit the proliferative cycle, which is in contrast to the expression activity of cdc2 and cdk2. In contrast to other cell division cycle kinases to which it is closely related, cdk5 appears not to be expressed in dividing cells in the developing brain. Ckd5 is a serine/threonine kinase which has approximately 60% structural identical to Cdc2 and Cdk2. (Tsai LH, Takahashi T, Caviness VS Jr, Harlow E., Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system., Development. 1993 Dec;119(4):1029-40) However, as shown in Exhibit A, Cdk5 from mouse, human and rat are virtually identical. Other publications identifying and describing Cdk5 existed in the art at the time the present invention was filed. They include:

A. Nikolic M, Dudek H, Kwon YT, Ramos YF, Tsai LH.
The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation.
Genes Dev. 1996 Apr 1;10(7):816-25.
PMID: 8846918 [PubMed - indexed for MEDLINE]

B. Songyang Z, Lu KP, Kwon YT, Tsai LH, Filhol O, Cochet C, Brickey DA, Soderling TR, Bartleson C, Graves DJ, DeMaggio AJ, Hoekstra MF, Blenis J, Hunter T, Cantley LC.
A structural basis for substrate specificities of protein Ser/Thr kinases: primary sequence preference of casein kinases I and II, NIMA, kinase, calmodulin-dependent kinase II, CDK5, and Erk1.
Mol Cell Biol. 1996 Nov;16(11):6486-93.
PMID: 8887677 [PubMed - indexed for MEDLINE]

C. Dhavan R, Tsai LH.
A decade of CDK5.
Nat Rev Mol Cell Biol. 2001 Oct;2(10):749-59. Review.
PMID: 11584302 [PubMed - indexed for MEDLINE]

4. At the time of filing of the present invention on February 19, 2002, a person of skill in the art was capable of easily recognizing a Dab1 protein and distinguishing it from other proteins, including the closely related Dab2 protein. Numerous publications identifying and describing Dab1 existed in the art. Howell et al., Mouse disabled (mDab1): a Src binding protein implicated in neuronal development. EMBO J. 1997 Jan 2;16(1):121-32 described mDab1 as a protein localized in the growing nerves of embryonic mice and as being tyrosine phosphorylated when the nervous system is developing, but not thereafter. This is in contrast to mDab2 which is widely expressed without any evidence of tyrosine phosphorylation or association with tyrosine-phosphorylated proteins. Howell et al., Dab1 tyrosine phosphorylation sites relay

positional signals during mouse brain development, Current Biology, Vol. 10:877-885 (2000) state that the identity between Dab1 and Dab2 in the PTB domain is 66%, in the DabH1 region is 73% and in the DabH2 region is 54%. However, as shown in Exhibit B, Dab1 amino acid sequences from mouse, rat and human are virtually identical throughout the entire protein. Other publications describing Dab1 and Dab2 include:

D. Xu XX, Yang W, Jackowski S, Rock CO.
Additions and Corrections to Cloning of a novel phosphoprotein regulated by colony-stimulating factor 1 shares a domain with the Drosophila disabled gene product.
J Biol Chem. 1996 May 31;271(22):13292. No abstract available.
PMID: 8663093 [PubMed - as supplied by publisher]

E. Xu XX, Yang W, Jackowski S, Rock CO.
Cloning of a novel phosphoprotein regulated by colony-stimulating factor 1 shares a domain with the Drosophila disabled gene product.
J Biol Chem. 1995 Jun 9;270(23):14184-91.
PMID: 7775479 [PubMed - indexed for MEDLINE]

F. Fulop V, Colitti CV, Genest D, Berkowitz RS, Yiu GK, Ng SW, Szepesi J, Mok SC.
DOC-2/hDab2, a candidate tumor suppressor gene involved in the development of gestational trophoblastic diseases.
Oncogene. 1998 Jul 30;17(4):419-24.
PMID: 9696034 [PubMed - indexed for MEDLINE]

G. Xu XX, Yi T, Tang B, Lambeth JD.
Disabled-2 (Dab2) is an SH3 domain-binding partner of Grb2.
Oncogene. 1998 Mar 26;16(12):1561-9.
PMID: 9569023 [PubMed - indexed for MEDLINE]

H. Sheldon M, Rice DS, D'Arcangelo G, Yoneshima H, Nakajima K, Mikoshiba K, Howell BW, Cooper JA, Goldowitz D, Curran T.
Scrambler and yotari disrupt the disabled gene and produce a reeler-like phenotype in mice.
Nature. 1997 Oct 16;389(6652):730-3.
PMID: 9338784 [PubMed - indexed for MEDLINE]

I. Howell BW, Hawkes R, Soriano P, Cooper JA.
Neuronal position in the developing brain is regulated by mouse disabled-1.
Nature. 1997 Oct 16;389(6652):733-7.
PMID: 9338785 [PubMed - indexed for MEDLINE]

J. Howell BW, Lanier LM, Frank R, Gertler FB, Cooper JA.
The disabled 1 phosphotyrosine-binding domain binds to the

internalization signals of transmembrane glycoproteins and to phospholipids.
Mol Cell Biol. 1999 Jul;19(7):5179-88.
PMID: 10373567 [PubMed - indexed for MEDLINE]

5. At the time of filing of the present invention on February 19, 2002, the term "Cdk5 serine kinase activity" was well understood and used extensively in the field. This statement refers to the ability of the enzyme (Cdk5) to transfer phosphate groups to specific serine residues present within a sequence of amino acid residues recognized by Cdk5.

6. Therefore at the time of filing of the present invention, a person of skill in the art was easily capable of distinguishing a Cdk5 or Dab1 protein from other proteins and could easily identify whether or not Cdk5 had serine kinase activity (e.g. was capable of phosphorylating serine).

7. Both Cdk5 and Dab1 proteins, especially the functional domains of each (the kinase domain of Cdk5 and the recognition sequence in Dab1) are highly conserved. Therefore, it is expected that the enzyme-substrate interaction is conserved among species that have a Cdk5 and Dab1 protein.

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

T. C

Thomas Curran

April 22, 2005
Date

Evidence Appendix
Exhibit 2

ProgramNumber:100.4 Day/Time:Saturday, Nov. 8, 4:00 PM -5:00 PM

Phospho -Dab1 is a specific biomarker for Cdk5 activation in developing and injured rodent brains.

W.Wang[1]; L.Keshvara[2]; T.Curran[2]; G.Zajic[1]; S.Jiao[1]; J.Louis[1]; C.M.Henley[1]; E.Magal[1*]

1. NeuroBiol., Amgen, Inc., Thousand Oaks, CA, USA; 2. Developmental Neurobiology, St. Jude's Children's Res. Hosp., Memphis, TN, USA

The cytoplasmic adapter protein Dab1 is related to the Drosophila disabled gene product. It is predominantly expressed in neurons and has been shown to function downstream of Reelin in a signaling pathway that controls laminar organization in the developing mammalian brain. Cyclin-dependent kinase 5 (Cdk5) and its neuron-specific activator p35 are also required for neurite outgrowth and cortical lamination. Biochemical cross-talk exists between these two (Reelin, Cdk5) signaling pathways that control cell positioning. Cdk5 phosphorylates Dab1 on specific serine sites in vivo, thus making it a specific biomarker for Cdk5 activation. We investigated Dab1 phosphorylation in the rat brain before (controls) and after ischemia-reperfusion injury (90 minutes of middle cerebral artery occlusion (MCAO) followed by reperfusion for 2 and 6h). Embryonic wild-type and Cdk-5 knockout mouse brains were used as positive controls, since the phospho-Ser 491 Dab1 antibody has been validated in these tissues (Keshvara, et al., J. Neuroscience, 22:4869-77, 2002). P-Dab1 was present in wild type mouse brain and absent in Cdk5 knockout mouse brains. The pSer491-Dab1 antibody specificity was demonstrated by lose of signal in pre-absorbed phospho-Dab1 peptide control. Phospho-Dab1 increased 1.7 to 11-fold, respectively, in the ipsilateral cortex at 2 and 6 hours after reperfusion in the MCAO rat model. Our studies indicate: 1) Dab1 phosphorylation on serine 491 is Cdk5 activity-dependent; 2) Dab1 antibody can be used as a specific marker for Cdk5 activation; 3) Increased phosphorylation of Dab1 is observed in the MCAO rat brain, validating CDK5 activity involvement in stroke pathology.

Citation:W.Wang, L.Keshvara, T.Curran, G.Zajic, S.Jiao, J.Louis, C.M.Henley, E.Magal. Phospho -Dab1 is a specific biomarker for Cdk5 activation in developing and injured rodent brains.. Program No. 100.4. 2003AbstractViewer/ItineraryPlanner.Washington, DC: Society for Neuroscience
ApplicationDesignandProgrammingCopyrightScholarOne,Inc.AllRightsReserved.PatentPending.

Evidence Appendix
Exhibit 3

Dabl Protein Alignment
Mouse, Rat and Human

```
MouseDabl       MSTETELQVAVKTSAKKDSRKKGQDRSEATLIKRFKGEGVRYKAKLIGIDEVSAARGDKL 60
RatDabl         MSTETELQVAVKTSAKKDSRKKGQDRSEATLIKRFKGEGVRYKAKLIGIDEVSAARGDKL 60
HumanDabl       MSTETELQVAVKTSAKKDSRKKGQDRSEATLIKRFKGEGVRYKAKLIGIDEVSAARGDKL 60
                ************************************************************

MouseDabl       CQDSMMKLKGVVAGARSKGEHKQKIFLTISFGGIKIFDEKTGALQHHHAVHEISYIAKDI 120
RatDabl         CQDSMMKLKGVVAGARSKGEHKQKIFLTISFGGIKIFDEKTGALQHHHAVHEISYIAKDI 120
HumanDabl       CQDSMMKLKGVVAGARSKGEHKQKIFLTISFGGIKIFDEKTGALQHHHAVHEISYIAKDI 120
                ************************************************************

MouseDabl       TDHRAFGYVCGKEGNHRFVAIKTAQAAEPVILDLRDLFQLIYELKQREELEKKAQKDKQC 180
RatDabl         TDHRAFGYVCGKEGNHRFVAIKTAQAAEPVILDLRDLFQLIYELKQREELEKKAQKDKQC 180
HumanDabl       TDHRAFGYVCGKEGNHRFVAIKTAQAAEPVILDLRDLFQLIYELKQREELEKKAQKDKQC 180
                ************************************************************

MouseDabl       EQAVYQTILEEDVEDPVYQYIVFEAGHEPIRDPETEENIYQVPTSQKKEGVYDVPKSQPV 240
RatDabl         EQAVYQTILEEDVEDPVYQYIVFEAGHEPIRDPETEENIYQVPTSQKKEGVYDVPKSQPV 240
HumanDabl       EQAVYQTILEEDVEDPVYQYIVFEAGHEPIRDPETEENIYQVPTSQKKEGVYDVPKSQPV 240
                ************************************************************

MouseDabl       SAVTQLELFGDMSTPPDITSPPTPATPGDAFLPAPSQTLPGSADVFGSMSPGTAAVPSGY 300
RatDabl         SAVTQLELFGDMSTPPDITSPPTPATPGDAFLPAPSQTLPGSADVFGSMSPGTAAVPSGY 300
HumanDabl       SAVTQLELFGDMSTPPDITSPPTPATPGDAFIPSSSQTLPASADVFSGVPFGTAAVPSGY 300
                ********************************:*;.*****.*****.*;.**********

MouseDabl       VAMGAVLPSFWGQQPLVQQQIAMGAQPPVAQVIPGAQFIAMGQPGLFPATQQPWPTVAGQ 360
RatDabl         VAMGAVLPSFWGQQPLVQQQIAMGAQPPVAQVIPGAQFIAMGQPGLFPATQQPWPTVAGQ 360
HumanDabl       VAMGAVLPSFWGQQPLVQQQMVMGAQPPVAQVMPGAQFIAMGQPGLFPATQQPWPTVAGQ 360
                **********************:.**********:******************************

MouseDabl       FPPAAFMPTQTVMPLPAAMFQGPLTPLATVPGTNQSARSSPQSQKPRQKMGKEMFKDFQM 420
RatDabl         FPPAAFMPTQTVMPLPAAMFQGPLTPLATVPGTNQSARSSPQSQKPRQKMGKEMFKDFQM 420
HumanDabl       FPPAAFMPTQTVMPLPAAMFQGPLTPLATVPGTSDSTRSSPQTQKPRQKMGKETFKDFQM 420
                ************************************.**:*****;********** *******

MouseDabl       AQPPPVPSRKPDQPSLTCTSEAFSSYFNKVGVAQDTDDCQDFDISQLMLTPVTSTTPSTN 480
RatDabl         AQPPPVPSRKPDQPSLTCTSEAFSSYFNKVGVAQDTDDCQDFDISQLMLTPVTSTTPSTN 480
HumanDabl       AQPPPVPSRKPDQPSLTCTSEAFSSYFNKVGVAQDTDDCQDFDISQLMLTPVTSTTPSTN 480
                ************************************************************

MouseDabl       SPFTPAPRQSSPSKSSASHVSDPTADDIFEEGFESPSKSEEQEAPDGSQASSTSDPFGEP 540
RatDabl         SPFTPAPRQSSPSKSSASHVSDPTADDIFEEGFESPSKSEEQEAPDGSQASSTSDPFGEP 540
HumanDabl       SPFTPAPRQSSPSKSSASHASDPTTDDIFEEGFESPSKSEEQEAPDGSQASSNSDPFGEP 540
                ********************.****;*******************************.*******

MouseDabl       SGEPSGDNISPQDGS 555
RatDabl         SGEPSGDNISPQDGS 555
HumanDabl       SGEPSGDNISPQAGS 555
                ************* **
```

Evidence Appendix
Exhibit 4

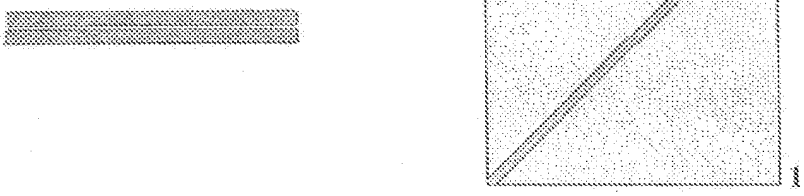
Blast 2 Sequences results

BLAST 2 SEQUENCES RESULTS VERSION BLASTP 2.2.13 [Nov-27-2005]

Matrix: BLOSUM62 gap open: 11 gap extension: 1
x_dropoff: 50 expect: 300.00 wordsize: 3 Filter: View option: Standard
Masking character option: X for protein, n for nucleotide Masking color option: Black
Show CDS translation Align

Sequence 1: gi|1771282|emb|CAA69662.1|mDab555 protein [Mus musculus]
Length = 555 (1 .. 555)

Sequence 2: gi|68533079|dbj|BAE06094.1|DAB1 variant protein [Homo sapiens]
Length = 559 (1 .. 559)



NOTE:Bitscore and expect value are calculated based on the size of the nr database.

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 Identities = 536/555 (96%), Positives = 543/555 (97%), Gaps = 0/555 (0%)

Query  1    MSTETELQVAVKTSAKKDSRKKGQDRSEATLIKRFKGEGVRYKAKLIGIDEVSAARGDKL  60
            MSTETELQVAVKTSAKKDSRKKGQDRSEATLIKRFKGEGVRYKAKLIGIDEVSAARGDKL
Sbjct  5    MSTETELQVAVKTSAKKDSRKKGQDRSEATLIKRFKGEGVRYKAKLIGIDEVSAARGDKL  64

Query  61   CQDSNMKLKGVVAGARSKGEHKQKIFLTISFGGIKIFDEKTGALQHRHAVHEISYIAKDI  120
            CQDSNMKLKGVVAGARSKGEHKQKIFLTISFGGIKIFDEKTGALQHRHAVHEISYIAKDI
Sbjct  65   CQDSNMKLKGVVAGARSKGEHKQKIFLTISFGGIKIFDEKTGALQHRHAVHEISYIAKDI  124

Query  121  TDHRAFGYVCGKEGNHRFVAIKTAQAAEPVILDLRDLFQLIYELKQREELEKKAQKDKQC  180
            TDHRAFGYVCGKEGNHRFVAIKTAQAAEPVILDLRDLFQLIYELKQREELEKKAQKDKQC
Sbjct  125  TDHRAFGYVCGKEGNHRFVAIKTAQAAEPVILDLRDLFQLIYELKQREELEKKAQKDKQC  184

Query  181  EQAVYQTILEEDVEDPVYQYIVFEAGHEPIRDPETEENIYQVPTSQKKEGVYDVPKSQPV  240
            EQAVYQTILEEDVEDPVYQYIVFEAGHEPIRDPETEENIYQVPTSQKKEGVYDVPKSQPV
Sbjct  185  EQAVYQTILEEDVEDPVYQYIVFEAGHEPIRDPETEENIYQVPTSQKKEGVYDVPKSQPV  244

Query  241  SAVTQLELFGDMSTPPDITSFPTPATPGDAFLPSSSQTLPGSADVFGSMSFGTAAVPSGY  300
            SAVTQLELFGDMSTPPDITSFPTPATPGDAF+PSSSQTLP SADVF S+ FGTAAVPSGY
```

Exhibit 4
page 2

```
Sbjct  245  SAVTQLELPGDMSTPPDIT  PTPATPGDAFIPSSSQTLPASADVFSSVPFC  AVPSGY  304

Query  301  VAMGAVLPSFWGQQPLVQQQIAMGAQPPVAQVIPGAQPIAMGQPGLFPATQQAWPTVAGQ  360
            VAMGAVLPSFWGQQPLVQQQ+ MGAQPPVAQV+PGAQPIAMGQPGLFPATQQ WPTVAGQ
Sbjct  305  VAMGAVLPSFWGQQPLVQQQMVMGAQPPVAQVMPGAQPIAMGQPGLFPATQQPWPTVAGQ  364

Query  361  FPPAAFMPTQTVMPLAAAMPQGPLTPLATVPGTNDSARSSPQSDKPRQKMGKESFKDFQM  420
            FPPAAFMPTQTVMPL AAMPQGPLTPLATVPGT+DS RSSPQ+DKPRQKMGKE+FKDFQM
Sbjct  365  FPPAAFMPTQTVMPLPAAMPQGPLTPLATVPGTSDSTRSSPQTDKPRQKMGKETFKDFQM  424

Query  421  VQPPPVPSRKPDQPSLTCTSEAFSSYFNKVGVAQDTDDCDDFDISQLNLTPVTSTTPSTN  480
             QPPPVPSRKPDQPSLTCTSEAFSSYFNKVGVAQDTDDCDDFDISQLNLTPVTSTTPSTN
Sbjct  425  AQPPPVPSRKPDQPSLTCTSEAFSSYFNKVGVAQDTDDCDDFDISQLNLTPVTSTTPSTN  484

Query  481  SPPTPAPRQSSPSKSSASHVSDPTADDIFEEGFESPSKSEEQEAPDGSQASSTSDPFGEP  540
            SPPTPAPRQSSPSKSSASH SDPT DDIFEEGFESPSKSEEQEAPDGSQASS SDPFGEP
Sbjct  485  SPPTPAPRQSSPSKSSASHASDPTTDDIFEEGFESPSKSEEQEAPDGSQASSNSDPFGEP  544

Query  541  SGEPSGDNISPQDGS  555
            SGEPSGDNISPQ GS
Sbjct  545  SGEPSGDNISPQAGS  559


CPU time:     0.05 user secs.       0.02 sys. secs          0.07 total secs.

Lambda     K      H
   0.311    0.129    0.372

Gapped
Lambda     K      H
   0.267   0.0410    0.140


Matrix: BLOSUM62
Gap Penalties: Existence: 11, Extension: 1
Number of Sequences: 1
Number of Hits to DB: 3330
Number of extensions: 1692
Number of successful extensions: 10
Number of sequences better than 300.0: 1
Number of HSP's gapped: 1
Number of HSP's successfully gapped: 1
Length of query: 555
Length of database: 1,196,146,007
Length adjustment: 138
Effective length of query: 417
Effective length of database: 1,196,145,869
Effective search space: 498792827373
Effective search space used: 498792827373
Neighboring words threshold: 9
X1: 16 ( 7.2 bits)
X2: 129 (49.7 bits)
X3: 129 (49.7 bits)
S1: 42 (21.8 bits)
S2: 68 (30.8 bits)
```

Evidence Appendix
Exhibit 5

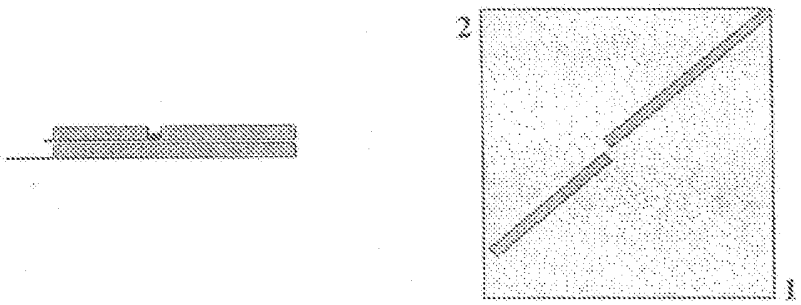
Blast 2 Sequences results

BLAST 2 SEQUENCES RESULTS VERSION BLASTP 2.2.13 [Nov-27-2005]

Matrix: BLOSUM62 gap open: 11 gap extension: 1
x_dropoff: 50 expect: 300.00 wordsize: 3 Filter: ☐ View option: Standard
Masking character option: X for protein, n for nucleotide Masking color option: Black
☐ Show CDS translation Align

Sequence 1: gi|1771282|emb|CAA69662.1|mDab555 protein [Mus musculus]
Length = 555 (1 .. 555)

Sequence 2: gi|73956392|ref|XP_852920.1|PREDICTED: similar to Disabled homolog 1 [Canis familiaris]
Length = 678 (1 .. 678)



NOTE:Bitscore and expect value are calculated based on the size of the nr database.

```
 Score = 1024 bits (2648),  Expect = 0.0
 Identities = 513/567 (90%), Positives = 520/567 (91%), Gaps = 33/567 (5%)

Query  22   KGQDRSEATLIKRFKGEGVRYKAKLIGIDEVSAARGDKLCQDSMMKLKGVVAGARSKGEH  81
            +GQDRSEATLIKRFKGEGVRYKAKLIGIDEVSAARGDKLCQDSMMKLKGVVAGARSKGEH
Sbjct  112  QGQDRSEATLIKRFKGEGVRYKAKLIGIDEVSAARGDKLCQDSMMKLKGVVAGARSKGEH  171

Query  82   KQKIFLFISFGGIKIFDEKTGALQHHSAVHEISYIAKDITDHRAFGYVCGKEGNERFVAI  141
            KQKIFLFISFGGIKIFDEKTGALQHHSAVHEISYIAKDITDHRAFGYVCGKEGNERFVAI
Sbjct  172  KQKIFLFISFGGIKIFDEKTGALQHHSAVHEISYIAKDITDHRAFGYVCGKEGNERFVAI  231

Query  142  KTAQAAEPVILDLRDLFQLIYELKQREELEKKAQKDKQCEQAVYQTILEEDVEDPVYQYI  201
            KTAQAAEPVILDLRDLFQLIYELKQREELEKKAQKDKQCEQAVYQTILEEDVEDPVYQYI
Sbjct  232  KTAQAAEPVILDLRDLFQLIYELKQREELEKKAQKDKQCEQAVYQTILEEDVEDPVYQYI  291

Query  202  VFEAGHEPIRDPETEENIYQVPTSQKKEGVYDVPKSQPVS----------------------  241
            VFEAGHEPIRDPETEENIYQVPTSQKKEGVYDVPKSQPVS
Sbjct  292  VFEAGHEPIRDPETEENIYQVPTSQKKEGVYDVPKSQPVSNGQPFEDFEERFAAATPNRN  351

Query  242  --------------AVTQLELFGDMSTPPDITSPPTPATPGDAFLPSSSQTLPGSADVPGS  288
                          AVTQLELFGDMSTPPDITSPPTPATPGDAF+PSSSQTLP SAD+P S
```

Exhibit 5
Page 2

```
Sbjct  352  LPMDFDEILEATKAVTQL  KDMSTPPDITSPPTPATPGDAFIPSSSQTL  ADMFSS  411

Query  289  MSFGTAAVPSGYVAMGAVLPSFWGQQPLVQQQIAMGAQPPVAQVIPGAQPIANGQPGLFP  348
            + FGTAAVPSGYVAMGAVLPSFWGQQPLVQQQIAMGAQPPVAQV+PGAQPIANGQPGLFP
Sbjct  412  VPFGTAAVPSGYVAMGAVLPSFWGQQPLVQQQIAMGAQPPVAQVMPGAQPIANGQPGLFP  471

Query  349  ATQQAWPTVAGQFPPAAFMPTQTVMPLAAAMFQGPLTPLATVPGTNDSARSSPQSDKPRQ  408
            ATQQ WPTVAGQFPPAAFMPTQTVMPL AAMFQGPLTPLATVP T DS RSSPQ++KPRQ
Sbjct  472  ATQQFWPTVAGQFPPAAFMPTQTVMPLPAAMFQGPLTPLATVPTTGDSTRSSPQTEKPPQ  531

Query  409  KMGKESFKDFQMVQPPPVPSRKPDQPSLTCTSEAFSSYFNKVGVAQDTDDCDDFDISQLN  468
            KMGKE FKDFQM QPPPVPSRNPDQPSLTCTSEAFSSYFNKVGVAQDTDDCDDFDISQLN
Sbjct  532  KMGKEMFKDFQMAQPPPVPSRKPDQPSLTCTSEAFSSYFNKVGVAQDTDDCDDFDISQLN  591

Query  469  LTPVTSTTPSTNSPPTPAPRQSSPSKSSASHVSDPTADDIFEEGFESPSKSEEQSAPDGS  528
            LTPVTSTTPSTNSPPTPAPRQSSPSKSSASH SDPT DDIFEEGFESPSKSEEQSAPDGS
Sbjct  592  LTPVTSTTPSTNSPPTPAPRQSSPSKSSASHASDPTTDDIFEEGFESPSKSEEQSAPDGS  651

Query  529  QASSTSDPFGEPSGEPSGDNISPQDGS  555
            QASS SDPFGEPSGEPSGDNISPQ GS
Sbjct  652  QASSNSDPFGEPSGEPSGDNISPQAGS  678
```

```
CPU time:     0.05 user secs.      0.01 sys. secs          0.06 total secs.

Lambda     K      H
   0.311    0.129    0.373

Gapped
Lambda     K      H
   0.267   0.0410    0.140


Matrix: BLOSUM62
Gap Penalties: Existence: 11, Extension: 1
Number of Sequences: 1
Number of Hits to DB: 3695
Number of extensions: 1958
Number of successful extensions: 11
Number of sequences better than 300.0: 1
Number of HSP's gapped: 1
Number of HSP's successfully gapped: 1
Length of query: 555
Length of database: 1,196,146,007
Length adjustment: 138
Effective length of query: 417
Effective length of database: 1,196,145,869
Effective search space: 498792827373
Effective search space used: 498792827373
Neighboring words threshold: 9
X1: 16 ( 7.2 bits)
X2: 129 (49.7 bits)
X3: 129 (49.7 bits)
S1: 42 (21.6 bits)
S2: 68 (30.6 bits)
```

Evidence Appendix
Exhibit 6

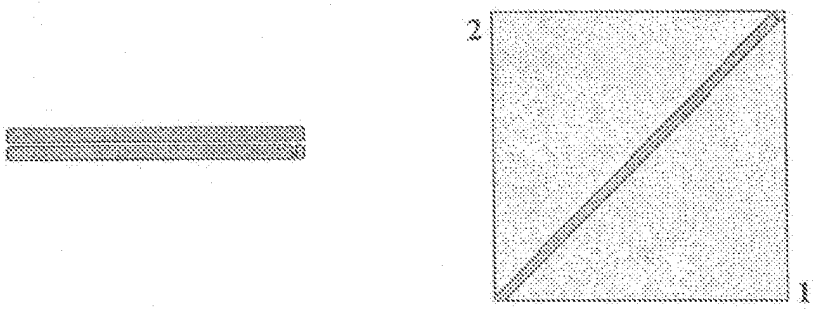
Blast 2 Sequences results

BLAST 2 SEQUENCES RESULTS VERSION BLASTP 2.2.13 [Nov-27-2005]

Matrix: BLOSUM62 gap open: 11 gap extension: 1
x_dropoff: 50 expect: 300.00 wordsize: 3 Filter: View option: Standard
Masking character option: X for protein, n for nucleotide Masking color option: Black
Show CDS translation Align

Sequence 1: gi|1771282|emb|CAA69662.1|mDab555 protein [Mus musculus]
Length = 555 (1 .. 555)

Sequence 2: gi|37933763|gb|AAP70754.1|disabled-1 late isoform [Gallus gallus]
Length = 551 (1 .. 551)



NOTE:Bitscore and expect value are calculated based on the size of the nr database.

```
 Score = 1013 bits (2620),  Expect = 0.0
 Identities = 497/555 (89%), Positives = 523/555 (94%), Gaps = 4/555 (0%)

Query  1    MSTETELQVAVKTSAKKDSRKKGQDRSEATLIKRFKGDGVRYKAKLIGIDEVSAARGDKL  60
            MSTETELQVAVKTS KKDS+KKGQDRSEATLIKRFKG+GVRYKAKLIGIDEVSAARGDKL
Sbjct  1    MSTETELQVAVKTSTKKDSKKKGQDRSEATLIKRFKGDGVRYKAKLIGIDEVSAARGDKL  60

Query  61   CQDSMMKLKGVVAGARSKGEHKQKIFLTISFGGIKIFDEKTGALQHHHAVHEISYIAKDI  120
            CQDSMMKLKG+VA ARSKGEHKQKIFLT+SFGGIKIFDEKTG LQHHHAVHEISYIAKDI
Sbjct  61   CQDSMMKLKGIVAAARSKGEHKQKIFLTVSFGGIKIFDEKTGLLQHHHAVHEISYIAKDI  120

Query  121  TDHRAFGYVCGKEGNHRFVAIKTAQAAEPVILDLRDLFQLIYELKQREELEKKAQKDKQC  180
            TDHRAFGYVCGKEGNHRFVAIKTAQAAEPVILDLRDLFQLIYELKQREE+EKKAQKDKQC
Sbjct  121  TDHRAFGYVCGKEGNHRFVAIKTAQAAEPVILDLRDLFQLIYELKQREEMEKKAQKDKQC  180

Query  181  EQAVYQTILEEDVEDPVYQYIVFEAGHEPIRDPETEENIYQVPTSQKKEGVYDVPKSQPV  240
            EQAVYQTILEEDVEDPVYQYIVFEAGHEPIR+PETEENIYQVPTSQKKEGVYDVPKSQPV
Sbjct  181  EQAVYQTILEEDVEDPVYQYIVFEAGHEPIREPETEENIYQVPTSQKKEGVYDVPKSQPV  240

Query  241  SAVTQLELFGGMSTPPDITSPPTPATPGDAFLPSSSQTLPGSADVFGSMSFGTAAVPSGY  300
            SAVTQLELFGGMSTPPD+TSPPTPATPGDAF+PSSSQ+LP S D+FGS+ F TAAVPSGY
```

Exhibit 6
page 2

```
Sbjct  241  SAVTQLELFGDMSTPPDVT  PTPATPGDAFIPSSSQSLPASTQMFGSVPFS  VPSGY  300

Query  301  VAMGAVLPSFWGQQPLVQQQIAMGAQPPVAQVIPGAQPIAWGQPGLFPATQQAWPTVAGQ  360
            VAMGAVLPSFWGQQPLVQQQ+AMGAQPPVAQV+ G QPIAWGQPG+FP  QQ WP+VAGQ
Sbjct  301  VAMGAVLPSFWGQQPLVQQQLAMGAQPPVAQVMQGGQPIAWGQPGIFPPAQQPWPSVAGQ  360

Query  361  FPPAAFMPTQTVMPLAAAMFQGGPLTPLATVPGTNDSARSSPQSDKPRQKMGKESEKDFQM  420
            F P AFMPTQTV+PL AAMFQG + P+ATVP T+DS RSSPQ+D+PRQKMGKE EKDFQM
Sbjct  361  FQPTAFMPTQTVLPLQAAMFQGTIAPIATVPPTSDSNRSSPQTDRPRQKMGKEMEKDFQM  420

Query  421  VQPPPVPSRKPDQPSLTCTSEAFSSYFNKVGVAQDTDDCDDFDISQLNLTPVTSTTPSTN  480
             QPPPVPSRKPIQPSL+CTSEAFSSYFNKVG+AQ+ DDCDDFDISQLNLTPVTSTTPSTN
Sbjct  421  AQPPPVPSRKPDQPSLSCTSEAFSSYFNKVGMAQEADDCDDFDISQLNLTPVTSTTPSTN  480

Query  481  SPPTPAPRQSSPSKSSASHVSDPTADDIFEEGFESPSKSEEQEAPDGSQASSTSDFFGEP  540
            SPPT  +QSSPSKSSASH SDP ADD+FEEGFESPSKSEEQEAPD SQASS SDFF
Sbjct  481  SPPTPAPRQSSPSKSSASHTSDPAADDLFEEGFESPSKSEEQEAPDESQASSNSDFF---  537

Query  541  SCEPSGDNISPQDGS  555
             CEP+GD ISPQ GS
Sbjct  538  -CEPTGDTISPQVGS  551
```

```
CPU time:     0.05 user secs.      0.01 sys. secs          0.06 total secs.

Lambda     K      H
   0.311    0.129    0.373

Gapped
Lambda     K      H
   0.267   0.0410    0.140

Matrix: BLOSUM62
Gap Penalties: Existence: 11, Extension: 1
Number of Sequences: 1
Number of Hits to DB: 3243
Number of extensions: 1668
Number of successful extensions: 10
Number of sequences better than 300.0: 1
Number of HSP's gapped: 1
Number of HSP's successfully gapped: 1
Length of query: 555
Length of database: 1,196,146,007
Length adjustment: 130
Effective length of query: 417
Effective length of database: 1,196,145,869
Effective search space: 498792827373
Effective search space used: 498792827373
Neighboring words threshold: 9
X1: 16 ( 7.2 bits)
X2: 129 (49.7 bits)
X3: 129 (49.7 bits)
S1: 42 (21.8 bits)
S2: 68 (30.8 bits)
```

Evidence Appendix
Exhibit 7

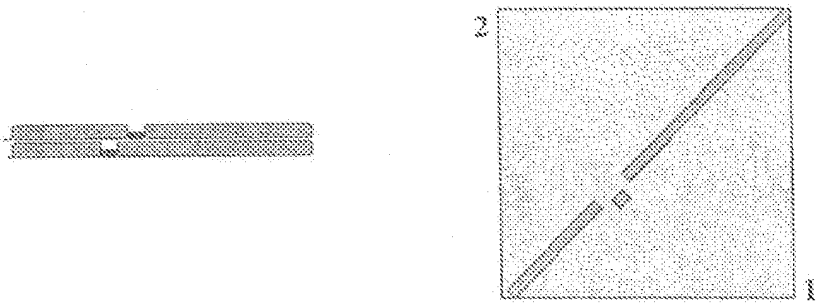
Blast 2 Sequences results

BLAST 2 SEQUENCES RESULTS VERSION BLASTP 2.2.13 [Nov-27-2005]

Matrix: BLOSUM62 gap open: 11 gap extension: 1
x_dropoff: 50 expect: 300.00 wordsize: 3 Filter: ☐ View option: Standard
Masking character option: X for protein, n for nucleotide Masking color option: Black
☐ Show CDS translation Align

Sequence 1: gi|1771282|emb|CAA69662.1|mDab555 protein [Mus musculus]
Length = 555 (1 .. 555)

Sequence 2: gi|76613795|ref|XP_582976.2|PREDICTED: similar to Disabled homolog 1 [Bos taurus]
Length = 542 (1 .. 542)



NOTE:Bitscore and expect value are calculated based on the size of the nr database.

```
 Score =  930 bits (2424),  Expect = 0.0
 Identities = 480/567 (84%), Positives = 485/567 (85%), Gaps = 68/567 (11%)

Query  22   KGQDRSEATLIKRFKGEGVRYKAKLIGIDEVSAARGDKLCQDSMMKLKGVVAGARSKGEH  81
            KGQDRSEATLIKRFKGEGVRYKAKLIGIDEVSAARGDKLCQDSMMKLKGVVAGARSKGEH
Sbjct  11   KGQDRSEATLIKRFKGEGVRYKAKLIGIDEVSAARGDKLCQDSMMKLKGVVAGARSKGEH  70

Query  82   KQKIFLTISFGGIKIFDEKTGALQHSSAVHEISYIAKDITDHRAFGYVCGKEGNSRFVAI  141
            KQKIFLTISFGGIKIFDEKTGALQHSSAVHEISYIAKDITDHRAFGYVCGKEGNSRFVAI
Sbjct  71   KQKIFLTISFGGIKIFDEKTGALQHSSAVHEISYIAKDITDHRAFGYVCGKEGNSRFVAI  130

Query  142  KTAQAAEPVILDLRDLFQLIYELKQREELEKKAQKDKQCEQAVYQTILEEDVEDPVYQYI  201
            KTAQAAEPVILDLRDLFQLIYELKQREELEKKAQKDKQCEQAVYQ
Sbjct  131  KTAQAAEPVILDLRDLFQLIYELKQREELEKKAQKDKQCEQAVYQ----------------  175

Query  202  VFEAGHEPIRDPSTEENIYQVPTSQKKEGVYDVPKSQPVS----------------------  241
                                 VPTSQKKEGVYDVPKSQPVS
Sbjct  176  ----------------------VPTSQKKEGVYDVPKSQPVSNGRAFEDFDERPAAATFNRN  215

Query  242  ----------------AVTQLELFGDMSTPPDITSPPTPATPGDAFLPSSSQTLPGSADVFGS  289
                           AVTQLELFGDMSTPPDITSPPTPATPGDAF+PSSSQTLP SADV GS
```

Exhibit 7 page 2

```
Sbjct  216  LPMDFDEIFEATKAVTQLF  GDMSTPPDITSPPTPATPGCAFIPSSSQTLF  KDVCGS  275

Query  289  MSFGTAAVPSGYVAMGAVLPSFWGQQPLVQQQIAMGAQPPVAQVIFGAQPIAWGQPGLFF  348
            + FGTAAVPSGYVAMGAVLPSFWGQQPLVQQQIAMGAQPPVAQV+FGAQPIAWGQPGLFP
Sbjct  276  LPFGTAAVPSGYVAMGAVLPSFWGQQPLVQQQIAMGAQPPVAQVMFGAQPIAWGQPGLFP  335

Query  349  ATQQAWPTVAGQFPPAAFMPTQTVMPLAAAMFQGPLTPLATVPGTWDSARSSPQSDKPRQ  408
            A QQ WP VAGQFPPAAFMPTQTVMPL AAMFQGPLTPLAT+P T DSARSSPQ+DKPRQ
Sbjct  336  AAQQPWPAVAGQFPPAAFMPTQTVMPLPAAMFQGPLTPLATIPATGDSARSSPQTDKPRQ  395

Query  409  KMGKESFKDFQMVQPPPVPSRKPDQPSLTCTSEAFSSYFNKVGVAQDTDDCDDFDISQLN  468
            KMGKE FKDFQM QPPPVPSRKPDQPSLTCTSEAFSSYFNKVGVAQDTDDCDDFDISQLN
Sbjct  396  KMGKEMFKDFQMAQPPPVPSRKPDQPSLTCTSEAFSSYFNKVGVAQDTDDCDDFDISQLN  455

Query  469  LTPVTSTTPSTNSPPTPAPRQSSPSKSSASHVSDPTADDIFEEGFESPSKSEEQEAPDGS  528
            LTPVTSTTPSTNSPPTPAPRQSSPSKSSASH SDPTADDIFEEGFESPSKSEEQEAPDGS
Sbjct  456  LTPVTSTTPSTNSPPTPAPRQSSPSKSSASHASDPTADDIFEEGFESPSKSEEQEAPDGS  515

Query  529  QASSTSDPFGEPSGEPSGDNISPQDGS  555
            QASS SDPFGEPSGEPSGDNISPQ GS
Sbjct  516  QASSNSDPFGEPSGEPSGDNISPQAGS  542
```

```
CPU time:     0.04 user secs.       0.01 sys. secs          0.05 total secs.

Lambda      K       H
   0.311    0.129    0.373

Gapped
Lambda      K       H
   0.267   0.0410    0.140

Matrix: BLOSUM62
Gap Penalties: Existence: 11, Extension: 1
Number of Sequences: 1
Number of Hits to DB: 3195
Number of extensions: 1648
Number of successful extensions: 13
Number of sequences better than 300.0: 1
Number of HSP's gapped: 1
Number of HSP's successfully gapped: 1
Length of query: 555
Length of database: 1,196,146,007
Length adjustment: 139
Effective length of query: 417
Effective length of database: 1,196,145,869
Effective search space: 498792827373
Effective search space used: 498792827373
Neighboring words threshold: 9
X1: 16 ( 7.2 bits)
X2: 129 (49.7 bits)
X3: 129 (49.7 bits)
S1: 42 (21.8 bits)
S2: 68 (30.8 bits)
```

Evidence Appendix
Exhibit 8

Blast 2 Sequences results

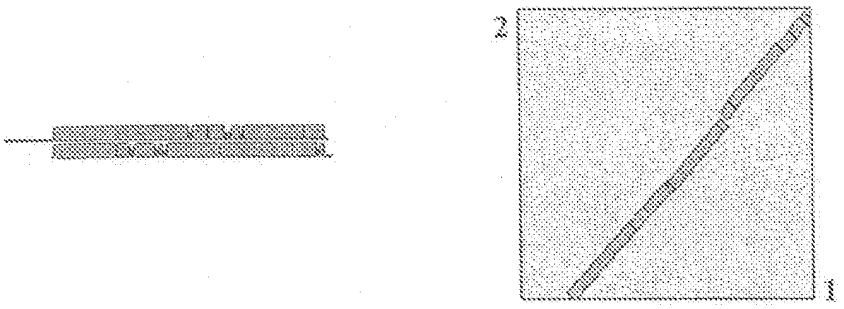
PubMed Entrez BLAST OMIM Taxonomy Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTP 2.2.13 [Nov-27-2005]

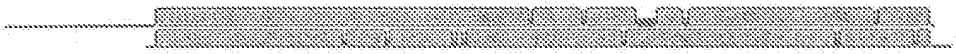
Matrix: BLOSUM62 gap open: 11 gap extension: 1
x_dropoff: 50 expect: 300.00 wordsize: 3 Filter: ☐ View option: Standard
Masking character option: X for protein, n for nucleotide Masking color option: Black
☐ Show CDS translation Align

Sequence 1: gi|1771282|emb|CAA69662.1|mDab555 protein [Mus musculus]
Length = 555 (1 .. 555)

Sequence 2: gi|68440873|ref|XP_686628.1|PREDICTED: similar to disabled homolog 1 [Danio rerio]
Length = 487 (1 .. 487)



NOTE: Bitscore and expect value are calculated based on the size of the nr database.



```
 Score =  505 bits (1309),  Expect = 2e-165
 Identities = 318/478 (66%), Positives = 359/478 (75%), Gaps = 34/478 (7%)

Query  97   FDEKTGALQHRHAVHEISYIAKDITDHRAFGYVCGKEGNHRFVAIKTAQAAEPVILDLRD  156
            F   +  LQHHHAVHEISYIAKDITDHRAFGYVCGKEGNHRFVAIKTAQ+AEPVILDLRD
Sbjct  8    FRRDSEVLQHHHAVHEISYIAKDITDHRAFGYVCGKEGNHRFVAIKTAQSAEPVILDLRD  67

Query  157  LFQLIYELKQREELEKKAQKDKQCEQAVYQTILEEDVEDPVYQYIVFEAGHEPIRQPETE  216
            LFQLIYE+KQREE+EKKAQKDKQCEQAVYQTILEEDVEDPVYQYIVFEAGHEPIRDP +E
Sbjct  68   LFQLIYEIKQREEIEKKAQKDKQCEQAVYQTILEEDVEDPVYQYIVFEAGHEPIRDP-SE  126

Query  217  ENIYQVPTSQKKEGVYDVPKSQPVGAVTQLELFGDMSTPPDITSPPTPATPGDAFLPSSS  276
            E+IYQVPTSQ+KEGVYDVPK  P    + QLELFGDMSTPPDITSP TPA+P +   P  +
Sbjct  127  ESIYQVPTSQQKEGVYDVPKRHP--NINQLELFGDMSTPPDITSPSTPASPANTLDPLLA  184

Query  277  QTLPGSADVFGSMSPGTAAVPSGYVAMGAVLPSFWGQQPLVQQQIAMGAQPF--VAQVIP  334
               P  +++F    F  A+VFSGYV MGAV P++  QQ   Q  +A G Q P  VAQV+P
Sbjct  185  HQTP--SELF--TPFNPASVPSGYVTMGAVPPAWAQQQFAAQAFLAFGVQSPVQVAQVLP  240

Query  335  GAQPIAWGQPGLFPATQQAWPTVAG-QFPPAAFMPTQTVMFLAAAMFQGELTPLA------  388
            G QP+ WGQ  LFPATQQ W   +AG   F PAAFMP QTV  L AAMFQ  L P+A
```

Exhibit 8 page 2

```
Sbjct  241  GTQPLIWGQANLFPATQC  \AMAGAHFSPAAFMPAQTVGPLPAAMPQ-TL   AVPASC  299

Query  389  ----------TVPGTNDSARSSPQSD-----KPRQKMGKESFKDFQMVQPPFVPSRKPDQPS  435
                      V GT+ S  SSPQ       + + KM KE FK+FQM +PP +P+RK +QPS
Sbjct  300  ETPTAAMGGAVAGTSASTASSPQHGERTLQRQAKMSKEMFKEFQMAKPPAMPARKGEQPS  359

Query  436  LTCTSEAFSSYFNKVGVAQDTDDCDDFDISQLNLTPVTSTTPSTNSPPTPAPRQSSPSKS  495
            L+CT++AFSSYF++VG+AQDTDDCDDFDISQ+NLTPVTSTTPSTNSPPTPAPRQSSPSKS
Sbjct  360  LSCTTDAFSSYFSRVGMAQDTDDCDDFDISQMNLTPVTSTTPSTNSPPTPAPRQSSPSKS  419

Query  496  SASHVSDPTADDIFEEGFESPSKS-EEQEAPDGSQASSTSDPFGEPSGEPSGDNISPQ  552
            S SR SDP  DD F E   SPS+S EE  A D  Q+   S+P  EP    S +  SPQ
Sbjct  420  S-SHASDPPTDDSPGEAEGSPSRSGEEDAAGDCPQSPGASEPQAEPE----SSETDSPQ  473
```

```
CPU time:     0.05 user secs.       0.01 sys. secs          0.06 total secs.

Lambda     K      H
   0.311    0.129    0.373

Gapped
Lambda     K      H
   0.267   0.0410    0.140


Matrix: BLOSUM62
Gap Penalties: Existence: 11, Extension: 1
Number of Sequences: 1
Number of Hits to DB: 2847
Number of extensions: 1507
Number of successful extensions: 18
Number of sequences better than 300.0: 1
Number of HSP's gapped: 1
Number of HSP's successfully gapped: 1
Length of query: 555
Length of database: 1,196,146,007
Length adjustment: 138
Effective length of query: 417
Effective length of database: 1,196,145,869
Effective search space: 498792827373
Effective search space used: 498792827373
Neighboring words threshold: 9
X1: 16 ( 7.2 bits)
X2: 129 (49.7 bits)
X3: 129 (49.7 bits)
S1: 42 (21.8 bits)
S2: 68 (30.8 bits)
```

Evidence Appendix
Exhibit 9

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0270-7306/96/$04.00+0
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A Structural Basis for Substrate Specificities of Protein Ser/Thr Kinases: Primary Sequence Preference of Casein Kinases I and II, NIMA, Phosphorylase Kinase, Calmodulin-Dependent Kinase II, CDK5, and Erk1

Z. SONGYANG,[1,2] KUN PING LU,[3] YOUNG T. KWON,[4] LI-HUEI TSAI,[5] ODILE FILHOL,[6] CLAUDE COCHET,[6] DEBRA A. BRICKEY,[7] THOMAS R. SODERLING,[7] CHERYL BARTLESON,[7] DONALD J. GRAVES,[7] ANTHONY J. DeMAGGIO,[8] MERL F. HOEKSTRA,[8] JOHN BLENIS,[3] TONY HUNTER,[3] AND LEWIS C. CANTLEY[1,2]*

Division of Signal Transduction, Beth Israel Hospital, Boston, Massachusetts 02215[1]; Molecular Biology and Virology Laboratory, The Salk Institute, La Jolla, California 92037[2]; Department of Cell Biology[3] and Department of Pathology,[4] Harvard Medical School, Boston, Massachusetts 02115; INSERM U244, DBMS/BRCE, CEN, Grenoble, France[5]; Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, Portland, Oregon 97201[6]; Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011[7]; and ICOS Corporation, Bothell, Washington 98021[8]

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We have developed a method to study the primary sequence specificities of protein kinases by using an oriented degenerate peptide library. We report here the substrate specificities of eight protein Ser/Thr kinases. All of the kinases studied selected distinct optimal substrates. The identified substrate specificities of these kinases, together with known crystal structures of protein kinase A, CDK2, Erk2, twitchin, and casein kinase I, provide a structural basis for the substrate recognition of protein Ser/Thr kinases. In particular, the specific selection of amino acids at the +1 and −3 positions to the substrate serine/threonine can be rationalized on the basis of sequences of protein kinases. The identification of optimal peptide substrates of CDK5, casein kinases I and II, NIMA, calmodulin-dependent kinases, Erk1, and phosphorylase kinase makes it possible to predict the potential in vivo targets of these kinases.

The essential role of protein kinases in regulating signal transduction was established with the discovery of cyclic AMP-dependent protein kinase (PKA) (12). To respond to different extracellular stimuli, distinct groups of protein kinases have evolved. Each protein kinase is thought to phosphorylate a unique set of targets in the cell. The substrate specificities of protein kinases are therefore crucial for the fidelity of signaling events.

The classical approach for studying the specificity of a protein kinase is to compare the phosphorylation kinetics of synthetic peptides on the basis of known sequences phosphorylated by the kinase. This procedure is helpful in identifying the amino acids critical for efficient phosphorylation. However, it is not practical to synthesize and study each of the billions of possible variations of sequences that must be considered. Moreover, it is extremely difficult to apply this approach to study the specificity of a protein kinase with no known substrates. To overcome these problems, we developed a method for determining the primary sequence specificities of protein kinases by using an oriented degenerate peptide library (21). Optimal peptide substrates of a given protein kinase are identified by phosphorylation of a pool of degenerate peptides containing billions of different species. The specificities determined for PKA, CDC2, and CDK2 by using this technique were consistent with known substrates of these kinases. The results also allowed the prediction of in vivo kinase substrates. Synthetic peptides based on predicted optimal motifs were shown to act as low-K_m substrates for the kinases studied. Therefore, this method is a useful tool for studying substrate specificities of protein kinases.

We present here the specificities of eight additional protein Ser/Thr kinases: CDK5, casein kinase I (CKI) δ and γ, casein kinase II (CKII), NIMA, calmodulin-dependent (Cam) kinase II, Erk1, and phosphorylase kinase. Our findings demonstrate that each of these protein kinases has a distinct optimal peptide substrate. Critical determinants for recognition by the protein kinases were found at residues both N and C terminal to the phosphorylation site. The selectivities of these kinases were also rationalized on a structural basis. Finally, the optimal substrate sequences identified can be used to predict in vivo targets of these protein kinases.

MATERIALS AND METHODS

Peptide libraries. The following Ser-oriented degenerate peptide library was used (21) for CKI and -II, Cam kinases I and II, NIMA, and phosphorylase kinase. Met-Ala-Xxx-Xxx-Xxx-Xxx-Ser-Xxx-Xxx-Xxx-Xxx-Ala-Lys-Lys-Lys, where Xxx indicates all amino acids except Trp, Cys, Tyr, Ser, and Thr. For the proline-directed protein kinase CDK5 and Erk1, the following Ser-Pro-oriented library was used: Met-Ala-Xaa-Xaa-Xaa-Xaa-Ser-Pro-Xaa-Xaa-Xaa-Ala-Lys-Lys-Lys, where Xaa stands for all amino acids except Cys and Trp. For CKI δ and γ, a Ser-Ile-oriented library (Met-Ala-Xaa-Xaa-Xaa-Xaa-Xaa-Ser-Ile-Xaa-Xaa-Xaa-Xaa-Ala-Lys-Lys-Lys) was also used.

Kinase reaction. CKII, Cam kinase II and the γ subunit of phosphorylase kinase were purified by conventional liquid chromatography. Glutathione *S*-transferase (GST)-CKI, GST-Erk1, and GST-NIMA were purified by using glutathione-agarose beads. p35/CDK5 complex was purified by immunoprecipitation using anti-CDK5 antibodies. The peptide library was phosphorylated by the protein kinase of interest in a buffer containing 100 μM ATP with a trace of [γ-^{32}P] ATP (roughly 6×10^5 cpm), 1 mM dithiothreitol, 10 mM $MgCl_2$, and 50 mM Tris (pH 7.5) as previously described (21). For Cam kinase II, Ca^{2+} (1 mM) and calmodulin (1 μM) were also added during the kinase reaction. In a typical reaction, the protein kinases (GST-CKI, GST-Erk1, and CKII [~10 ng], NIMA [~1 ng],

* Corresponding author. Mailing address: Division of Signal Transduction, Beth Israel Hospital, 330 Brookline Ave., Boston, MA 02115. Phone: (617) 667-0947. Fax: (617) 667-0957.

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TABLE 1. Substrate specificities of protein kinases

Ser kinase	Selectivity value										
	−5	−4	−3	−2	−1	0	+1	+2	+3	+4	+5
Arg/Lys directed											
Phosphorylase kinase (FRMMSFFLF)		F (1.7) M (1.5) K (1.4)	**R** (5.7) **K** (2.5)	**M** (3.0) R (1.8) Q (1.7) F (1.6)	**M** (2.2) F (2.0) L (1.8) I (1.5)	S	**F** (3.9) **I** (2.4) M (1.7) L (1.6)	**F** (2.5) **R** (2.0) K (2.0)	L (2.1) I (2.0)	F (2.3) L (1.8) I (1.7)	
Cam kinase II (KRQQSFDLF)		**K** (2.3) F (1.5)	**R** (3.9) **K** (2.7)	**Q** (4.5) M (1.8)	**Q** (2.1) **M** (2.0) K (1.9) L (1.8) F (1.5)	S	**F** (4.8) I (1.9) M (1.7) L (1.6) V (1.3)	**D** (2.5) R (1.7) I (1.5)	L (1.7) M (1.7) K (1.6) I (1.5)	F (1.7) K (1.4)	
Negative charge directed											
CKI δ (EFDTGSHIFF)	E (1.8)	F (1.7) E (1.6)	**D** (5.7)	T (1.9) A (1.8) G (1.8)	G (1.8)	S	I	I (2.5) F (1.8) Y (1.8) G (1.7)	L (1.9) G (1.7) F (1.6)	F (2.1) G (1.6)	F (2.2) F (1.7) L (1.5)
CKI γ (YYDAASHIFF)	Y (1.5)	Y (1.6) E (1.5)	**D** (2.7) **Y** (2.3)	A (3.0) D (2.0)	A (1.6) G (1.6)	S	I	I (2.8) Y (2.2) F (2.0)	L (1.9) F (1.6) Y (1.5)	F (2.6) Y (1.8)	F (2.1)
CKII (EDEESEDEE)		E (2.2) D (1.8) A (1.8)	D (3.2) E (2.2)	E (2.8) **D** (2.6)	E (2.7) **D** (2.0)	S	E (3.0) **D** (2.9) A (1.5)	**D** (3.4) E (2.3) A (1.6)	E (3.4) **D** (3.0)	E (4.1) **D** (2.1)	
Ser-Pro directed											
Erk1 (TGPLSPGPF)		T (2.6) P (1.5) S (1.4)	G (3.2) P (1.5) E (1.5) Y (1.5)	P (6.5) L (2.1) I (1.3)	L (2.0) M (1.9) F (1.7)	S	P	G (2.1) P (1.9) F (1.3)	P (2.4) F (1.9) G (1.5) Y (1.5)	F (2.3) Y (1.7) I (1.2)	
CDK5 (KHIHKSPKHR)		K (1.8) H (1.6) G (1.5)	H (1.6)	H (2.5) P (2.1)	K (2.0) G (1.7) H (1.5)	S	P	K (2.9) R (2.9)	H (4.5) R (4.0) K (3.9)	K (3.2) H (3.2) R (2.5)	
Hydrophobic amino acid directed											
NIMA (RFRRSRRMI)		R (2.0) N (1.5)	F (7.3) L (2.3) M (1.7)	R (4.5) K (1.2)	R (4.6) K (1.6)	S	R (2.6) I (1.7) V (1.7) M (1.7)	R (2.0) I (2.0) M (1.9) V (1.6)	M (1.9) I (1.8) F (1.7) V (1.5)	I (2.1) F (1.7) M (1.7)	

[a] Amino acids with selectivity values higher than 1.5 or the best two at every degenerate position are shown. Abbreviations for amino acid residues: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; Y, Tyr. Boldface letters indicate amino acids which were most preferentially selected (value of >2.0).

Cam kinases I and II, and phosphorylase kinase γ subunit (~20 μg)] were added to 300 μl of solution containing 1 mg of degenerate peptide mixture. GSP-Erk1 was activated by MEK in vitro before phosphorylation of the peptide library.

Phosphopeptide purification, sequencing, and data analysis. The phosphopeptides were separated from ATP by using a DEAE-Sephacel column and further purified from unphosphorylated peptides on an iron-chelating column (21). In this study, a relatively smaller iron column (300 μl instead of 1 ml as used previously [21]) was used such that unphosphorylated peptide contamination was significantly reduced. Peptide sequencing and data analysis were previously described (21).

RESULTS

Using the soluble oriented peptide library approach, we determined the optimal nonapeptide motifs for eight different protein Ser/Thr kinases. These enzymes were selected for study since they cover a broad spectrum of the known protein Ser/Thr kinases. The results are presented in Table 1. The selectivity values in parentheses indicate how strongly a particular amino acid is selected at a given position. A comparison of optimal substrate sequences of the protein Ser/Thr kinases studied (Table 1 and reference 21a) indicated that the −3 and +1 positions of the substrate play a major role in substrate recognition by most of the protein kinases. On the basis of these observations and previous studies by us and others, the protein Ser/Thr kinases studied can be classified in regard to their substrate specificity. A large number of protein Ser/Thr kinases (e.g., the Cam kinase family, PKC family, phosphorylase kinase γ, PKA, and S1,K1) strongly prefer an Arg/Lys residue at the −3 position and are thus named Arg/Lys-directed protein kinases. In contrast, protein Ser/Thr kinases such as CKI and CKII select for substrates with acidic residues or phosphorylated amino acids at the −3 and other positions. Some protein Ser/Thr kinases are proline-directed kinases in that they select for substrates with Pro at the +1 position. These include mitogen-activated protein kinases and cyclin-dependent kinases. Finally, the protein kinase NIMA belongs to a unique family which preferentially selects a hydrophobic residue at the −3 position.

Arg/Lys-directed protein kinases. (i) Optimal substrates for phosphorylase kinase. Phosphorylase kinase regulates glycogen metabolism by phosphorylating phosphorylase. Although very few substrates have been reported for phosphorylase kinase, this kinase is an interesting enzyme which has the ability to phosphorylate serine, threonine, and, under certain conditions, tyrosine residues in vitro (24). We examined its specificity in phosphorylating serine-containing sequences by using the γ subunit (kinase domain) of this kinase. As shown in Table 1, this kinase preferred peptides with the motif Phe-Arg-Met-Met-Ser-Phe-Phe-Leu-Phe. This optimal peptide sequence is consistent with known sites phosphorylated by this kinase (Table 2).

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TABLE 2. Comparison of optimal sequences determined by the peptide library with sequences at the phosphorylation sites of known protein substrates

Peptide or protein	Sequence[a]	Reference
Cam kinase II		
Consensus from library	K R Q Q S F D G P	
	F E M M I E M K	
Known sites		
Autophosphorylation	S R Q E T V D C L	17
Autophosphorylation	K R Q E T V E C L	17
Synapsin I	T R Q A S Q S G Q	17
Synapsin I	T R Q T S V S G Q	17
Tyrosine hydroxylase	G R P Q S L I P S	17
Calcineurin	A R V P S V L R R	17
Phospholamban	R R A S T I S M P	17
ATP-citrate lyase	A R T A S P S P S	17
Phosphorylase kinase		
Optimal sequence from library	F E M K R F P L F	
	R R R P I R I L	
Known substrate or site		
Phosphorylase	R K Q I S V R G L	17
Autophosphorylation	K R S S E I Y S P	17
CKI		
Optimal motif from library	S P E T G S I I P P	
	Y S Y A R Y G Y P	
Known substrates		
αs2-Casein	S*S*S*E S S I I S Q S	17
β-Casein	S*S*S*E S S I T R I S	17
Glycogen synthase	T L S*V S S L P G L S	17
Phosphorylase kinase β	T K P S S S I T K P L	17
CKII		
Optimal motif from library	S D S S S S D S S	
	D S D D D S D D	
Some known sites		
Nucleolar protein B23	S D A S S D S D	17
Human α-Hsp90	S S V G S D S S K	17
PKA subunit $R_{II\beta}$	A D S S S D D S S	17
Phosphatase inhibitor 2	S Q S S S G S S D	17
p35/CDK5		
Consensus from library	K H K K S P K K K	
	K P G S K P K	
	K K	
Known sites		
Rat neurofilament	K S T K S P V K K	19
	V S V K S P S K A	19
Simian virus 40 large T antigen	A G S A T P P K K	19
Erk1		
Consensus from library	T G S L S P G P P	
	P P L M P P Y	
Known sites (Erk1 or Erk2)		
Myelin basic protein	V T P R T P P P G	17
TAL1	M V Q L S P P A L	3
Tyrosine hydrolase	R A V T S P R F I	9
Epidermal growth factor receptor	V S P L T P S G S	22
Myc	T P P L S P S P R	6
Jun	T P P L S P I G R	6
Oncoprotein 18	R L I G S P R G K	2
Caldesmon	D K V T S P T K V	1
Caldesmon	D G N R S P A P K	1
NIMA		
Optimal sequence from library	R P R R S P R R I	
	G K P I P	
Known site (phospholemman)	T P R S S I K P G	15

[a] Residues in boldface are strongly selected, and underlined residues are more strongly selected. S* indicates phosphoserine.

(ii) Substrate specificity of Cam kinase II. Like phosphorylase kinase, PKA, and PKC, Cam kinase II preferred Arg at the −3 position and hydrophobic amino acids at the +1 position (Table 1). Cam kinase I had a substrate specificity similar but not identical to that of Cam kinase II (data not shown). Both Cam kinase I and Cam kinase II had unusually strong selections for Phe at the +1 position compared with other kinases studied. However, Cam kinase I differed from Cam kinase II in that it did not strongly select for substrates with Gln at the −2 position or for acidic amino acids (Asp and Glu) at the +2 position (data not shown). Some known phosphorylation sites of Cam kinase II are presented in Table 2. They are in good agreement with the peptide library results.

Negative charge-directed protein kinases. (i) Substrate specificities of CKI γ and δ. Two different peptide libraries were used to investigate the specificities of CKI γ and δ. Using a Ser-oriented library with degenerate amino acids at four positions on either side of the fixed Ser, an apparent selection for Ile at +1 was observed for both of these enzymes (data not shown). To further examine substrate preference of CKI isomers, a Ser-Ile fixed peptide library was used. As shown in Table 1, the optimal substrates predicted for CKI γ and δ are nearly identical. Both kinases strongly select peptides with a negatively charged residue (Asp) at the −3 position. It has been reported that CKIs prefer to phosphorylate Ser or Thr residues in regions of proteins that have been previously phosphorylated (3). Although phosphorylated amino acids are excluded from libraries used in these studies, the strong selection for Asp at the −3 position suggests a preference for substrates with phosphorylated amino acids at this position. Interestingly, there was no selection for peptides with acidic residues C terminal of the fixed Ser, suggesting that these kinases are likely to phosphorylate residues C terminal rather than N terminal of previously phosphorylated sites. Some known phosphorylation sites of CKI are listed in Table 2.

(ii) Substrate specificity of CKII. In agreement with previous peptide phosphorylation studies, CKII was found to preferentially phosphorylate peptides enriched in Glu and Asp (Table 1). In fact, Glu and Asp were preferred over other amino acids at all eight positions of degeneracy investigated. There was some position specificity in that Asp was strongly selected over Glu at the −3 and +2 positions whereas Glu was preferred over Asp at the +3 and +4 positions. In addition, acidic amino acids were more strongly selected at positions C terminal of Ser than at positions N terminal. Like CKIs, there is evidence that CKII phosphorylates regions of proteins that have been previously phosphorylated (14). Our results predict that while CKIs are more likely to phosphorylate C terminal of a previously phosphorylated region, CKII is more likely to phosphorylate N terminal of a previously phosphorylated site and CKII is less specific about the position of the negatively charged residue. Some known sites phosphorylated by CKII are presented in Table 2.

Pro-directed kinases. (i) Substrate specificity of p35/CDK5. CDK5, a member of the cyclin-dependent kinases, preferentially phosphorylated a library containing a fixed Ser-Pro motif, indicating a strong preference for Pro at the +1 position. The optimal substrate predicted for p35/CDK5 (Lys-His-His-Lys-Ser-Pro-Lys-His-Arg) is in close agreement with the sites that it phosphorylates on neurofilament proteins (19) (Table 2). This motif is similar to those that we previously obtained for cyclin A/CDK2 and cyclin B/CDC2 (21). These results indicate that the three kinases may share some common in vivo targets and that discrimination among potential in vivo targets is largely controlled by contacts outside the catalytic cleft. Our attempts to model peptide substrates into the crystal structure

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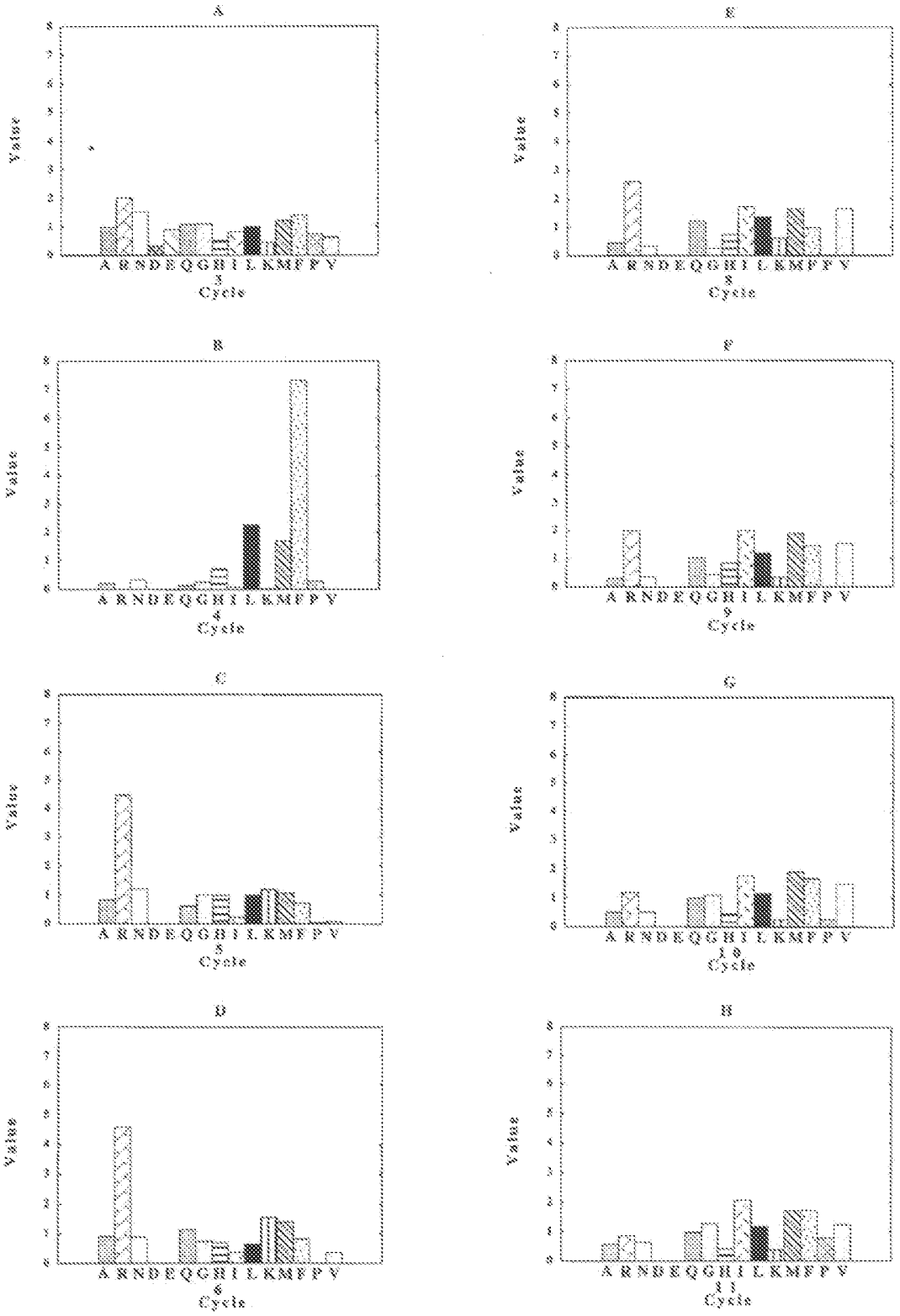

FIG. 1. Substrate specificity of protein kinase NIMA. Each panel indicates the relative abundance of the 15 amino acids at a given cycle of sequencing. Panels A to D indicate amino acid preferences at positions −4, −3, −2, and −1 N terminal of the phosphorylation site, and panels E to H indicate preferences at positions +1, +2, +3, and +4 C terminal of the phosphorylation site. Abbreviations for amino acid residues: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; Y, Tyr.

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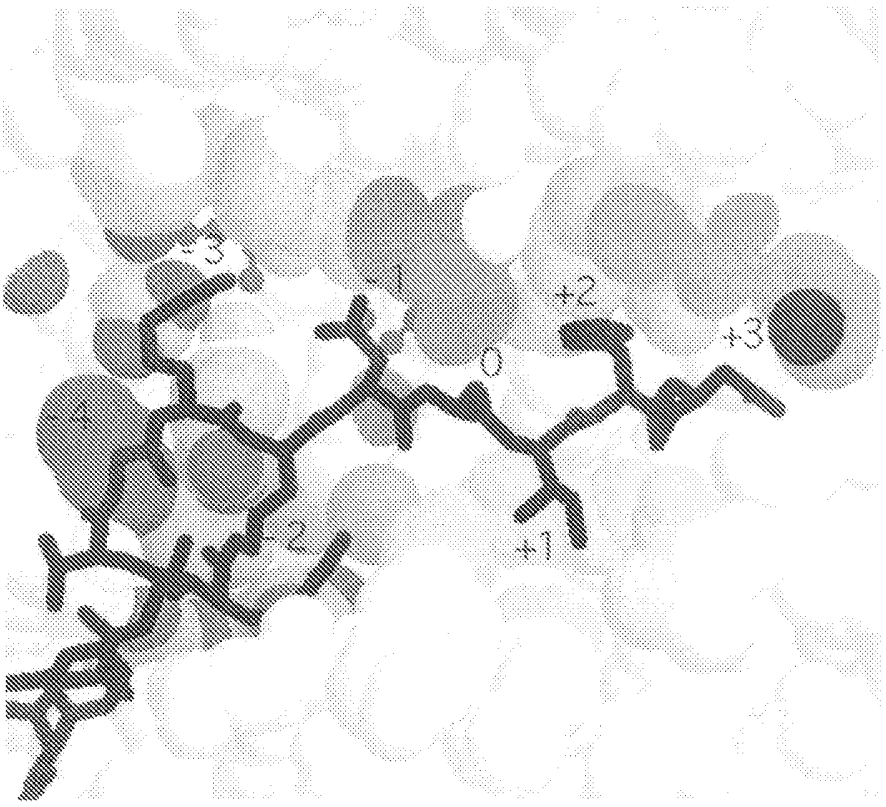

FIG. 2. Peptide substrate recognition by PKA. The catalytic cleft of PKA is presented with the pseudo-substrate, PKI (black) bound, on the basis of the PKI-PKA cocrystal (13). Those atoms in PKA that are proximal to the side chains of residues −4 to +3 of PKI [Gly-Arg-Arg-Asn-Ala(β)-Ile-His-Asn] are colored as follows: −4 pocket, violet; −3, orange; −2, pink; −1, green; +1 yellow; +2, orange; +3, cyan. The carboxylate oxygens of Glu and Asp residues in PKA that form the −4, −3, and −2 pockets are red, and an ε-nitrogen of Lys in PKA at the +3 pocket is blue.

of CDK2 (21) or the cyclin A/CDK2 (12) complex suggest that only amino acids that are more than four residues C terminal of the phosphorylated Ser are likely to directly contact the cyclin moiety. Libraries are being constructed to explore specificity at positions further from the catalytic cleft in order to determine the influence of the cyclin subunit in substrate selection.

(ii) Substrate specificity of Erk1. Like the cyclin-dependent kinases, Erk1 preferentially phosphorylated the Ser-Pro-oriented library. The optimal peptide substrate predicted for Erk1 was Thr-Gly-Pro-Leu-Ser-Pro-Gly-Pro-Phe (Table 1). Consistent with previous studies (6), peptides containing Pro at the −2 position were strongly preferred. Table 2 lists some sites known to be phosphorylated by Erk kinases. In close agreement with our prediction, five of the six known substrates have Leu at the −1 position. At the −2 position, hydrophobic amino acids, especially Pro, are frequently found. In fact, several known substrates of Erk1 have the motif PLS/TP (Table 2). Interestingly, the optimal motif is similar to consensus motifs that are known to bind SH3 domains in a proline-helix conformation.

Phe/hydrophobic amino acid-directed kinases. (i) Substrate specificity of NIMA. NIMA, a protein Ser/Thr kinase essential for G_2-M progression in *Aspergillus nidulans* (15, 16), was shown to have a unique optimal peptide substrate. Although genetic evidence exists for the importance of this kinase in cell growth regulation, in vivo substrates have not been identified. Using the peptide library, the optimal substrate is predicted to be Arg-Phe-Arg-Arg-Ser-Arg/hydrophobic-Arg/hydrophobic-hydrophobic-hydrophobic (Table 1). The human NIMA-like kinase (NLK1) had a similar substrate preference (not shown). The residue that dominates selectivity is the Phe at −3, which is distinct from the optimal motif of any previously characterized protein kinase. The strong selection for Phe at the −3 position is in agreement with in vitro substrates identified for NIMA (reference 15 and Table 2). The complete data for a selectivity experiment with this kinase are presented in Fig. 1 to elucidate the strong selection for Phe at the −3 position (cycle 4). To test the library data, we synthesized the optimal peptide (GRFRRSRRMI) for NIMA. This peptide is a good substrate for NIMA (K_m = 40.9 μM), with a higher V_{max}/K_m value than a peptide (GTFRSSIRRL) from previous best substrate, phospholemman (15).

DISCUSSION

On the basis of similar crystal structures observed for PKA, CDK2, Erk1, CKI, twitchin, and insulin receptor (4, 10, 11, 13, 23, 25) and homologous primary sequences (7, 8), it is likely that most of the eukaryotic protein kinases have similar tertiary structures. The structure of the PKA-PKI cocrystal established the first model for how a substrate binds to the active site of a protein kinase (13). The basis for the amino acids selected at specific positions N terminal and C terminal of the phosphor-

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POSITION/SUBSTR	POCKET			-4		POCKET			-3				POCKET					-2		POCKET	-1	
SUBDOMAIN	V					V	VI					VI	VIII			XI				I		
RESIDUE NO.	197	198	228			223	226	230				270	281	283	284	289	296			52	53	
ARG/LYS-DIRECTED KINASES																						
PKA/	D	K	D	ARG		S	S	T	ARG			D	T	S	Y	S	P	ARG		S	S	ARG/A+2
PKC ALPHA/	D	Q	D	ALA*		S	S	F	ARG*			D	T	S	Y	S	P	LYS*		S	S	ALA*
PKC EPSILON/	S	Q	D	LYS*		S	S	S	ARG*			D	T	S	Y	S	S	ALA*		S	S	ALA*
CAM KIN2 ALPHA/	S	V	K	LYS		S	S	S	ARG			S	T	S	Y	T	P	ALA		S	A	ALA
PHK GAMMA/	S	T	D	PHE		S	S	Y	ARG			S	T	S	Y	S	P	MET/ARG		S	V	MET
PHE-DIRECTED KINASE																						
NIMA/	D	K	R	ARG		S	S	W	PHE			S	T	P	Y	S	P	ARG		S	S	ARG
PRO-DIRECTED KINASES																						
CDC2/	P	S	-	HIS		S	S	-	HIS			S	S	W	Y	S	P	HIS/PRO		S	S	LYS
CDK2/	S	S	-	HIS		S	S	-	HIS			S	T	W	Y	S	P	HIS/PRO		S	S	ARG
CDK5/	S	S	-	LYS		S	S	-	HIS			S	T	W	Y	S	P	HIS/PRO		S	S	LYS
ERK1/	P	L	P	PRO		S	S	T	GLN			S	T	W	Y	S	P	PRO		S	K	LEU
GLU/ASP-DIRECTED KINASES																						
CKI GAMMA/	L	L	S	TYR		L	S	R	ASP			S	T	R	F	Y	L	ALA		S	S	ALA
CKI DELTA/	L	P	S	PHE		L	S	S	ASP			S	T	N	Y	T	L	TYR		S	S	GLY
CK2 ALPHA/	T	K	T	GLU		T	S	T	ASP			K	S	Y	P	S	P	GLN		S	K	GLN

POSITION/SUBSTRATE			POCKET				+3		POCKET			+2		POCKET	+3
SUBDOMAIN	VIA		VIII		X				I	II-III				II-III	
RESIDUE NO.	197	198	202	205	247				53	54	83	86		81	82	83	
ARG/LYS-DIRECTED KINASES																	
PKA/	F	L	P	L	Y	TLE		S	F	L	S	ILE		R	L	K	PHE/A+2
PKC ALPHA/	R	F	P	Y	P	LEU*		S	P	S	S	ARG*		S	S	S	ALA*
PKC EPSILON/	R	F	P	L	P	VAL*		S	P	S	S	ARG*		S	S	S	ARG*
CAM KIN2 ALPHA/	L	F	S	L	Y	PHE		A	F	R	S	ASN		R	N	S	LEU/LYS
PHK GAMMA/	F	V	P	L	L	PHE		V	S	L	S	PHE/ARG		S	L	R	LEU
PHE-DIRECTED KINASE																	
NIMA/	L	K	P	N	V	ARG/ILE		A	F	T	L	ARG/ILE		S	S		MET
PRO-DIRECTED KINASES																	
CDC2/	L	S	L	R	F	PRO		S	K	S	V	ARG		S	L	S	ARG
CDK2/	L	L	L	R	I	PRO		S	Y	S	V	ARG		S	R	S	LYS
CDK5/	L	S	L	R	F	PRO		S	K	S	V	ARG		S	L	S	HIS
ERK1/	L	Y	K	R	L	PRO		A	Y	S	Y	GLN		R	L	S	PRO
GLU/ASP-DIRECTED KINASES																	
CK2 GAMMA/	L	L	A	K	Y	ILE		S	F	S	R	ILE		F	L	L	ILE
CKI DELTA/	L	L	A	A	Y	ILE		S	F	S	R	ILE		F	L	L	ILE
CK2 ALPHA/	L	R	F	K	I	GLU		K	Y	F	K	ASP		F	F	V	GLU

FIG. 3. Protein kinase residues predicted to contact side chains of peptide substrates. Single-letter codes are used to indicate the residues of various protein kinases that are predicted to contact the side chains of the −4 to +3 residues of bound peptide substrates. The top row indicates the residues of PKA proximal to the −4 to +3 residues of PKI (Fig. 2). The arabic numbers indicate the positions of the residues in the linear sequence of PKA. The roman numerals designate the subdomain locations of the indicated residues (using the numbering system of Hanks et al. [8]). The analogous residues in the other protein kinases are based on sequence alignments (8) or, in some cases, on known crystal structures (see text). Three-letter codes are used to indicate the amino acids preferentially selected at the −4 to +3 positions for each of the kinases, using the oriented peptide library approach. The results for CDC2, CDK2, and PKA are from reference 21. For PKCα and PKCε, the sequences of pseudosubstrates, rather than optimal substrates, are included. Basic residues are blue, acidic residues are red, hydrophilic, uncharged residues are green, and hydrophobic residues (including Tyr) are black. A dash indicates that the kinase terminates prior to this residue. A question mark indicates uncertainty about alignment. An asterisk indicates a residue from an intrinsic pseudosubstrate rather than an optimal substrate.

ylation site can be rationalized from this structure. The PKA-PKI cocrystal is presented in Fig. 2. The atoms from PKA that are within 6 Å (0.6 nm) of the β-carbons of residues −4 through +3 of PKI are indicated in colors with different colors indicating distinct pockets for side chains.

The identities of the residues from PKA that form the various side chain pockets are summarized in the top row of Fig. 3. Figure 3 also indicates the subdomain locations of the residues, using the nomenclature of Hanks et al. (8). Single-letter codes indicate the residues that form the pockets, and three-letter codes indicate the optimal amino acid selected at each pocket by the oriented peptide library approach. The residues predicted to make up the −4 to +3 pockets of the various protein kinases in Fig. 3 were assigned on the basis of sequence alignments with the analogous positions in PKA or, in the cases of CDK2 (4, 12), CKI (23), and phosphorylase kinase (12a), information from crystal structures. The protein kinases have been divided into Arg/Lys-directed, Pro-directed, Phe-directed, and Glu/Asp-directed on the basis of the selectivities observed at the −3 or +1 positions, as discussed in Results.

A quick scan of the colors in Fig. 3 reveals some general trends. Those pockets with multiple acidic residues (red) usually select for basic (blue) amino acids (Arg/Lys/His). This is particularly clear for the −4, −3, and −2 pockets of the Arg/Lys-directed kinases. The cyclin-dependent kinases are also acidic (although less so) in the −4, −3, and −2 pockets and weakly selected for substrates with His at these positions. In contrast, the casein kinases have basic residues in the −3 pocket and selected for Asp at this position.

The explanation for the strong selectivity of NIMA for substrates with Phe at −3 is not completely clear. The predicted −3 pocket, like that of PKA, has two acidic residues. A possible explanation for the selectivity is the presence of a Trp residue at the analogous position of Tyr-330 of PKA in this region (Fig. 3). The Trp may provide the opportunity for aromatic ring interactions to stabilize Phe binding to this region.

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Similarities between NIMA and PKA in the regions that bind Arg at −2 have been previously discussed (15).

Most kinases investigated showed relatively low selectivity at the −1 position (Table 1). Side chains at the −1 position are predicted to interact with residues in subdomain I (e.g., residues 52 and 53 of PKA [Fig. 3]). Most kinases have small hydrophilic, uncharged residues at these positions. Interestingly, CKII has a Lys in this region and selects for Glu at −1, and phosphorylase kinase has a Val and selects for Met (Fig. 3). Some of the kinases selected for Arg/Lys at the −1 position, probably as a result of electrostatic attractions from residues in the nearby −3 pocket (Fig. 1).

As we previously discussed (21), the +1 pocket is very important for selectivity as well as for orienting the adjacent Ser/Thr residue for phosphorylation. This pocket is well separated from the other pockets and is entirely composed of residues between subdomains VII and VIII, near the C-terminal end of the T loop (yellow in Fig. 2). An examination of Fig. 3 reveals that most of the kinases that we have studied have very hydrophobic +1 pockets, and these kinases select for substrates with hydrophobic amino acids at +1. We previously discussed the prediction that the presence of an Arg residue at the base of this pocket (analogous to the Leu-205 position of PKA) results in selectivity for substrates with Pro at the +1 position (21). This prediction is based on modeling studies showing that the Arg side chain fills up the pocket and excludes residues with large side chains (or water molecules). Pro may also be selected at this position because it is the only naturally occurring amino acid that is a secondary amine. Thus, peptides with Pro at +1 can bind to this site without the necessity of dissociating a hydrogen-bonded water molecule from the amide nitrogen of the peptide (21). All Pro-directed protein kinases that we have investigated to date have Arg at this position. However, since several other residues are necessary to form the pocket, it is not clear that an Arg at this position alone is sufficient to predict selectivity for Pro at +1.

CKII is the only kinase that we have investigated that has a Lys residue at the base of the +1 pocket (Fig. 3). Consistent with this pocket being basic, CKII selects substrates with Glu at the +1 position.

A few of the kinases that we have investigated have strong selectivities at the +2 and/or +3 positions. For example, the cyclin-dependent kinases strongly select for substrates with basic residues at these positions (Table 1). Consistent with these selectivities, these kinases have acidic residues in the +2 and +3 pockets. Certain PKC family members have been shown to prefer substrates with basic residues at the +2 and +3 positions, and these kinases also have acidic pockets to account for these selectivities (Fig. 3). In contrast, CKII has basic residues in the +2 and +3 pockets and selects for substrates with Asp and Glu at these positions. Thus, while the presentation in Fig. 3 cannot explain every selectivity that we observed, it certainly provides explanations for most of the strong selectivities observed in Table 1.

In summary, we have determined the primary sequence specificities of eight protein Ser/Thr kinases by using an oriented peptide library technique. The results are in good agreement with known substrates of these kinases and should allow predictions of novel phosphorylation sites from primary sequences. On the basis of these results and alignments of these kinases with PKA, we have proposed a structural basis for substrate selectivities of these and other protein kinases. These predictions will guide future mutational studies to test the proposed models.

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Evidence Appendix
Exhibit 10

Sequence Revision History

PubMed Nucleotide Protein Genome Structure PMC Taxonomy OMIM

Find (Accessions, GI numbers or Fasta style SeqIds) x76104

About Entrez

Show difference between I and II as GenBank/GenPept

Entrez

Search for Genes
LocusLink provides curated information for human, fruit fly, mouse, rat, and zebrafish

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LinkOut

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BLAST

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Revision history for X76104

GI	Version	Update Date	Status	I	II
2094872	1	Aug 4 2003 7:22 PM	Live	◉	○
2094872	1	Oct 13 2002 6:12 PM	Dead	○	○
2094872	1	Mar 9 1999 1:52 AM	Dead	○	○
2094872	1	May 14 1997 12:11 PM	Dead	○	○
434846	0	May 28 1996 11:34 PM	Dead	○	◉
434846	0	May 23 1995 6:07 PM	Dead	○	○
434846	0	Feb 27 1995 12:05 AM	Dead	○	○
434846	0	Nov 30 1994 6:03 PM	Dead	○	○
434846	0	Dec 15 1993 12:21 AM	Dead	○	○

Accession X76104.1 was first seen at NCBI on Dec 15 1993 12:21 AM

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X. *RELATED PROCEEDINGS APPENDIX*

None